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EFFECT OF A DIETARY LYSINE DEFICIENCY ON THE CONCENTRATION OF AMINO ACIDS IN THE DEPROTEINIZED BLOOD PLASMA OF CHICKS[1]

JEAN A. GRAY,[2] ELLEN M. OLSEN, D. C. HILL, AND H. D. BRANION

Abstract

A lysine-deficient basal diet, both with and without supplemental lysine added, was fed to groups of Barred Plymouth Rock cockerels from hatching to 4 weeks of age. At this time blood samples were taken from the carotid artery and pooled for each group. Twelve amino acids were determined in the deproteinized plasma by microbiological assay. Deproteinized plasma from birds receiving the lysine-deficient diet was lower in lysine content and higher in threonine and tyrosine than that from birds fed the lysine-supplemented diet. Other amino acids showed much smaller differences. A similar amino acid pattern was observed when the birds were fed the lysine-supplemented diet for 25 days from hatching and then the lysine-deficient diet for 3 days before blood samples were taken. Withholding feed for 24 hours before the samples were taken resulted in a marked increase in lysine and threonine concentration, an effect which has been reported by other workers. Acid hydrolysis of the deproteinized plasma increased somewhat the concentration of lysine, as measured microbiologically, but had no effect on, or somewhat reduced, the concentrations of other amino acids.

Introduction

Studies in our laboratory (1, 2) have shown that a dietary deficiency of lysine produces, in addition to depressed growth, achromatosis of the feathers, reduced tyrosinase activity in the feather quills, and a macrocytic anemia. We have been interested in studying in more detail the relationship which a dietary deficiency of lysine bears to the production of these symptoms and also to possible other metabolic abnormalities. An investigation of the effect of a lysine deficiency in the diet on the concentration of amino acids in the plasma has been a part of this study.

Charkey *et al.* (3, 4) and Richardson *et al.* (5, 6) have studied amino acid concentration in chick plasma but these studies were limited to only a few amino acids and did not refer specifically to lysine-deficient diets.

Materials and Methods

In all experiments reported here Barred Plymouth Rock cockerels were used. Birds were housed in battery-type brooders and fed the experimental

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Contribution from the Department of Nutrition, Ontario Agricultural College, Guelph, Ontario. This article is based on a paper presented at the 41st Annual Conference and Exhibition of the Chemical Institute of Canada, Toronto, May 26–28, 1958.
[2]Present address: Vankleek Hill, Ontario.

diets ad libitum from the time they were 1 day old to the termination of the experiment.

The lysine-deficient diet used was of a practical type based on commercial sunflower seed oil meal manufactured by the expeller process. It consisted of ground wheat 50%, ground yellow corn 8.56%, sunflower seed oil meal 34.19%, soybean oil 1.0%, dehydrated grass 2.0%, dried buttermilk 2.0%, ground limestone 0.75%, steamed bone meal 1.25%, iodized salt 0.25% with the following per kilogram of diet, manganous sulphate 309 mg, riboflavin 8.6 mg, B_{12}-penicillin supplement (containing 3.0 mg vitamin B_{12} and 2.0 g procaine penicillin per lb) 4.3 g, dry concentrate of vitamin D_3 (16, 500 I.C.U. per g) 68.1 mg, and dry concentrate of vitamin A (250,000 I.U. per g) 33.7 mg. The protein content (N × 6.25) of this diet was approximately 24.0% and the lysine content by microbiological assay was 0.65%.

The lysine-adequate diet used was the diet just described plus L-lysine added at a level of 0.5% (7). This supplement was added in the form of a commercial preparation containing 95% of L-lysine monohydrochloride.

Blood samples for amino acid analysis were taken from the carotid artery. Three to four milliliters of blood was collected from each bird and pooled for each experimental group, which consisted of 25 to 40 birds depending on the experiment. Heparin was used as an anticoagulant. Following centrifugation the resulting plasma was deproteinized by the addition of tungstic acid according to the method of Hier and Bergeim (8). The protein-free filtrate was adjusted to pH 6.8 by adding 1 drop of 15% KOH solution with a final adjustment using 0.1 *N* KOH. The adjusted filtrates were stored under toluene at 5° C until analyzed.

Twelve amino acids were determined directly on the filtrates by microbiological methods. Three organisms were used: *Lactobacillus plantarum* for valine, *Streptococcus faecalis* 9780 for arginine, leucine, methionine, threonine, and trytophan, and *Leuconostoc mesenteroides* P60 for glycine, histidine, isoleucine, lysine, phenylalanine, and tyrosine. Assays were conducted using the assay medium of Henderson and Snell (9) with modifications described by Klain *et al.* (1).

Data for glutamic acid are not given in this report since assays for this amino acid by the methods used gave unsatisfactory results. Presumably relatively large amounts of glutamine, present in the plasma, interfered with the glutamic acid assay. Substantial amounts of glutamine were shown to be present in chicken plasma by paper chromatography. Samples of plasma were desalted on cation exchange columns, the eluted amino acids spotted on paper, and chromatograms obtained by the method of Levy and Chung (10), which employs the system of butanol–acetic acid–water followed by 1:1 *m*-cresol–phenol, pH 9.3 borate buffer. This finding is of interest since there are conflicting reports concerning the presence of glutamine in chicken plasma (11, 12, 13).

Recoveries of purified amino acids added to chick plasma before precipitation of the plasma proteins with tungstic acid were considered satisfactory. Percentage recoveries, given as the average of two experiments, the results of

which agreed closely, were as follows: lysine 104, threonine 107, tyrosine 98, arginine 98, glycine 98, histidine 99, valine 101, isoleucine 107, leucine 102, phenylalanine 98, tryptophan 93, methionine 97.

Results and Discussion

Effect of Feeding a Lysine-Deficient Diet for 4 Weeks

Two experiments were conducted. In the first experiment each diet was fed to two lots of 25 chicks. At the conclusion of the feeding period of 4 weeks, blood samples were taken immediately from one lot per diet and from the remaining lots 24 hours following the removal of feed. Assays were conducted on pooled samples for each lot. In the second experiment each diet was fed to four lots of 20 birds and blood samples were taken immediately from two lots per diet and from the remaining lots after a 24-hour fast.

Table I shows the levels of amino acids found in the plasma for experiments 1 and 2. The most marked changes observed in amino acid level were among

TABLE I

Effect of feeding a lysine-deficient diet for 4 weeks on the concentration of amino acids (mg%) in the plasma of chicks

	Full fed*				Fasted for 24 hours†			
	Lysine-adequate		Lysine-deficient		Lysine-adequate		Lysine-deficient	
Amino acid	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Lysine	4.6	4.1	1.0	1.2	10.9	11.6	9.3	8.9
Threonine	6.1	6.6	15.5	16.4	9.1	11.6	14.7	13.9
Tyrosine	2.4	2.5	3.8	4.1	2.3	2.4	2.7	2.9
Arginine	9.6	9.1	6.8	6.4	4.2	4.6	1.8	2.3
Glycine	5.1	4.9	5.5	5.1	4.5	4.5	4.1	4.1
Histidine	2.9	3.0	2.9	3.1	1.5	1.5	1.4	1.6
Valine	5.5	5.7	5.3	5.5	4.1	5.0	3.3	4.0
Isoleucine	2.5	2.4	2.1	2.3	2.4	2.7	1.8	1.8
Leucine	3.7	3.6	3.1	3.2	4.1	4.5	3.0	3.2
Phenylalanine	2.4	2.6	2.8	3.1	1.7	1.9	1.6	1.8
Tryptophan	1.8	2.0	1.5	1.6	1.2	1.4	1.0	1.1
Methionine	2.3	2.7	1.8	2.0	1.4	1.6	1.0	1.2

*Chicks were fed ad libitum for 4 weeks.
†Chicks were fed ad libitum for 4 weeks then the feed removed for 24 hours before blood samples were taken.

the first three amino acids listed, lysine, threonine, and tyrosine. Among the full-fed groups the removal of the lysine supplement from the diet resulted in a decrease in the lysine level, but an increase in the threonine and tyrosine levels. The other amino acids were affected only to a slight degree. The statistical significance of the differences observed for lysine, threonine, and tyrosine when the birds were full-fed was tested by pooling the results from the two experiments of Table I, additional results from feeding the same diets given below in Table II, and results from a fourth experiment not reported in detail here. The levels (mg%) of amino acids found in this latter experiment were as follows for the lysine-adequate and lysine-deficient diets respectively, lysine 5.8, 1.2; threonine 7.7, 18.5; tyrosine 2.1, 4.0. The observed differences pooled for the four experiments were highly significant ($P<1\%$). The decrease

in the lysine level is likely a direct reflection of the decreased amount of lysine in the feed being consumed. On the other hand, the increase in threonine and tyrosine is not so readily explained.

TABLE II

Effect of alteration in diet on the concentration of amino acids (mg%) in deproteinized plasma

Diet sequence	Lysine	Threonine	Tyrosine	Arginine
Lysine-adequate (A) for 4 weeks	4.7	7.0	2.6	9.8
Lysine-deficient (D) for 4 weeks	1.3	14.9	4.4	8.0
(A) for 2 weeks (D) for 2 weeks	1.7	16.0	4.3	12.5
(A) for 3 weeks (D) for 1 week	1.7	16.7	4.1	11.1
(A) for 25 days (D) for 3 days	1.6	17.1	4.4	9.9
(D) for 25 days (A) for 3 days	3.9	9.2	3.4	9.4

It will be seen from the data of Table I that depriving the birds of feed for 24 hours before sampling increased the level of lysine in the plasma regardless of the previous diet, and also increased the level of threonine when the diet was adequate in lysine. Other amino acids were either relatively unaffected by the fasting treatment or reduced somewhat in level.

Charkey *et al.* (3, 4) found similarly elevated levels of lysine and threonine in plasma of chicks subjected to a 48-hour fast. They postulated that lysine and threonine are less readily deaminated in the chick than are other amino acids, and consequently accumulate in the plasma when tissue protein or storage protein releases amino acids during the period of starvation. A similar hypothesis might be used to explain the elevated threonine level in the plasma when chicks were consuming a lysine-deficient diet. Under this regimen, utilization of amino acids for protein synthesis was restricted by the lysine deficiency, and the excess amino acids, including threonine, coming from the diet had to be disposed of. Threonine, if relatively difficult to deaminate, could thus accumulate in the plasma. However, it is doubtful if the elevated tyrosine level found when the chicks consumed the lysine-deficient diet could be explained in this way since the tyrosine level in the plasma decreased when the chicks were fasted.

All groups of birds receiving the lysine-deficient diet showed marked feather achromatosis (1) and it is tempting to suggest that the elevated level of tyrosine in the plasma was closely related metabolically to the abnormal feather pigmentation in the birds. However, such a relationship, if any, is obscure.

Effect of Period of Lysine Deficiency

In previous experiments groups of birds were fed continuously on the two diets from the time they were 1 day old until they were 4 weeks old. This was done primarily because a study of feather achromatosis was combined

with the study of plasma amino acids, and continuous feeding of a lysine-deficient diet to 4 weeks was necessary to produce severe lack of feather pigment.

It would seem likely, however, that the blood composition should reflect the diet composition quite rapidly, within a few days or hours. Consequently, an experiment was conducted in which the lysine-deficient diet was substituted for the lysine-adequate diet at 2 weeks, 3 weeks, and 25 days, and the blood sampled at 4 weeks and pooled for each group as before. The lysine-adequate diet (A) and the lysine-deficient diet (D) were fed for 4 weeks to lots of 40 birds divided into pens of 20 birds each. The other diet combinations were fed to lots of 25 birds.

Results for four amino acids are given in Table II.

The data from the continuous feeding of the two diets for 4 weeks are in good agreement with those presented in Table I. It also appears that feeding diet D for 3 days was sufficient to produce the characteristic elevation of threonine and tyrosine and depression of lysine. On the other hand, the reverse procedure of feeding diet D for 25 days, then diet A, appeared to result in only a partial restoration of lysine, threonine, and tyrosine to levels given by the continuous feeding of diet A for 4 weeks.

One may presume that, following the addition of lysine to the lysine-deficient diet, some time must elapse before a more normal rate of tissue synthesis can be reached and thus remove the excess levels of threonine and tyrosine which have accumulated in the blood plasma. On the other hand, an almost immediate rise in lysine at least to the level given by continuously feeding diet A, might have been anticipated when supplemental lysine was added. It is possible that this did not occur because the extended period of lysine deficiency resulted in a depletion of reserves of this amino acid. A considerable portion of the dietary lysine may have been diverted to fill these reserves.

Effect of Acid Hydrolysis of Deproteinized Plasma

It is known that assay microorganisms may utilize, not only free amino acids, but certain peptides and possibly amino acid conjugates (14, 15, 16). Therefore, it cannot be assumed that the assays, as used in this study, measured only the concentration of uncombined amino acids in the plasma. In the hope of obtaining some information on this problem, concentrations of amino acids in the plasma were compared before and after acid hydrolysis of the protein-free filtrates.

Filtrates obtained from experiment 1 were hydrolyzed as follows. A 10-ml aliquot of filtrate was evaporated just to dryness on a steam bath. The residue was transferred to a test tube using a few milliliters of water and sufficient concentrated HCl added to provide an approximately 2.5 *N* solution of acid. The tube was then sealed and heated at 115° C for 16 hours. The hydrolyzate was adjusted to pH 4.5 with KOH and filtered, and the filtrate adjusted to pH 6.8 and diluted to 50 ml.

The results are set forth in Table III.

TABLE III

Effect of acid hydrolysis on the concentration of amino acids (mg %) in deproteinized chick plasma

	Lysine-adequate, full-fed		Lysine-adequate, fasted		Lysine-deficient, full-fed		Lysine-deficient, fasted	
Amino acid	F	HF	F	HF	F	HF	F	HF
Lysine	4.6	6.3	10.9	13.0	1.0	2.4	9.3	10.8
Threonine	6.1	5.9	9.1	8.2	15.5	15.6	14.7	13.2
Arginine	9.6	9.4	4.2	4.3	6.8	6.4	1.8	1.7
Glycine	5.1	5.7	4.5	5.0	5.5	5.8	4.1	4.2
Histidine	2.9	2.8	1.5	1.7	2.9	2.6	1.4	1.6
Valine	5.5	5.4	4.1	3.6	5.3	5.0	3.3	3.1
Isoleucine	2.5	2.4	2.4	2.3	2.1	2.1	1.8	1.7
Leucine	3.7	3.6	4.1	3.7	3.1	2.7	3.0	2.5
Phenylalanine	2.4	2.3	1.7	1.6	2.8	2.6	1.6	1.4
Methionine	2.3	2.3	1.4	1.4	1.8	1.7	1.0	1.0

NOTE: Symbols F and HF stand for filtrate and hydrolyzed filtrate respectively.

With the exception of lysine which increased in concentration, and to a lesser extent glycine, hydrolysis of the plasma filtrate either had little effect on the amino acid levels or resulted in a small decrease. This finding is in contrast to a report by Henderson *et al.* (17) that hydrolysis of protein-free filtrates of rat plasma gave increases of 25 to 400% in amino acids concentrations as measured microbiologically, with most of the amino acid increasing about 200%. With respect to the data in Table III it seems unlikely that they were influenced by appreciable destruction of amino acids during the hydrolysis procedure since recoveries of lysine, leucine, phenylalanine, and threonine added to the plasma filtrate before hydrolysis were within the narrow range of 96 to 101%.

Certainly it is evident from Table III that the relationship between dietary lysine deficiency and fasting on the one hand, and plasma levels of lysine and threonine on the other, was similar for both hydrolyzed and unhydrolyzed plasma filtrates. Thus it would seem that the changes in amino acid level observed in the first two experiments could not be attributed to the synthesis or breakdown of peptides or amino acid conjugates.

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THE EFFECT OF VARIOUS DENATURANTS ON THE HAEMOGLOBINS OF ADULT AND CORD BLOOD

II. UREA AS A DENATURANT[1]

F. D. WHITE, AUDREY KERR, R. B. FERGUSON, AND A. R. HULL

Abstract

The action of urea upon the haemoglobins of human adult and cord blood has been studied spectrophotometrically under varying conditions of temperature, urea concentration, and pH, and the velocity constants of the reaction determined at different temperatures and at different molar concentrations of urea.

Breaks appeared in the reaction curves of adult blood at all temperatures studied and at urea concentrations greater than 4 *M*, but not in the case of cord blood, the corresponding rates of which are much lower than those of adult blood. It is suggested that these breaks may be indicative of the formation of an intermediate product, and only become apparent when the reaction attains a critical velocity, determined by conditions of temperature or urea concentration.

The product of the urea reaction at pH 8.5, and that of alkali denaturation at pH 11.9, both give absorption curves whose dissimilarity in shape has been shown to be due to the difference in pH. The absorption curve of alkaline globin haematin (the product of alkali denaturation) when adjusted to pH 8.5, is indistinguishable from that produced by the urea reaction. It is therefore concluded that since the products of these reactions are spectroscopically identical, the action of urea upon oxyhaemoglobin appears to be a true denaturation.

Introduction

In a previous communication (1) it was shown that the reactions of strong concentrations of urea and certain other denaturants with human oxyhaemoglobins were spectrophotometrically similar to that of alkali denaturation, in that there was a progressive drop in optical density at 578 mμ. In every case, however, the rate of reaction was markedly lower, the rate for urea (the slowest acting) having a velocity constant approximately one thousandth that of the sodium hydroxide denaturation of adult blood. There was also a break in the reaction curves indicating a change in velocity, while in the case of urea, the product of the reaction did not give the absorption curves typical of alkali denaturation either before or after reduction with sodium dithionite. Since urea is a well-known denaturant it seemed of interest to investigate further the reaction with haemoglobins under varying experimental conditions. For comparative purposes these reactions had all been carried out previously under the same conditions of temperature and pH, while the denaturant was used in the highest concentration consistent with complete solubility of the reactants (in the case of urea, about 7 *M*). Accordingly, the influence of temperature was first investigated, then the effect of progressively decreasing the molarity of the urea solution, and finally the effect of increasing the pH. The results now reported indicate that the rate of reaction varies with temperature in accordance with the Arrhenius relationship, that the break in the reaction curve is only apparent when the urea concentration is greater than 4 *M*, and

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Contribution from the Department of Biochemistry, University of Manitoba, Winnipeg, Manitoba, Canada. This work was aided by a grant from the National Research Council of Canada.

that the product of the reaction gives a spectrophotometric curve whose shape varies with the pH value.

Experimental

General Procedure

For the preparation of the blood samples, the procedure as outlined previously (1) was usually adhered to, although on many occasions the stroma was first removed by toluene and the appropriate amount of haemolyzate added to the Sørensen glycine–NaCl–NaOH buffer. Since, however, the removal of stroma did not appear to have any significant effect upon the reaction curve, even under conditions of varying molarity of the urea, this modification was not persevered with. A large number of experiments were carried out at each temperature and at each different urea concentration, and the results reported are typical of the values obtained.

(1) *The Effect of Variations in Temperature*

The denaturation of adult oxyhaemoglobin by sodium hydroxide at pH 11.9 is a first-order reaction, and shows the Arrhenius relationship between the logarithm of the velocity constant and the reciprocal of the absolute temperature.

To obtain the energy of activation it is therefore only necessary to determine the velocity constants at two different temperatures, and this has been done for human adult oxyhaemoglobin by Haurowitz *et al.* (2), who have also recorded values for the heat and entropy of activation. Nevertheless it seemed advisable to plot the relationship at several different temperatures for both adult and fetal blood, with a view to comparing the resultant graphs with the corresponding ones for the reaction with urea.

The denaturation of adult and cord blood by sodium hydroxide was carried out as previously described (3), the reaction being followed in a Beckman DU spectrophotometer at a wavelength of 578 mμ. In this series of experiments, the temperature varied from 4° to 26.5° C for adult blood and 7° to 24° C for cord blood, and was kept constant throughout each experiment by means of a Beckman thermospacer.

For the reaction with urea, the haemoglobin solution was mixed with five times its volume of urea solution, and samples of the mixture, which was maintained at a constant temperature, transferred to the Beckman cuvette at 15-minute intervals. Experiments with both adult and cord blood were carried out between 23° and 40° C.

For each reaction and at each temperature, the percentage of unchanged haemoglobin (H) at time t is represented by the equation

$$H = 100\ (D_t - D_0)\ /\ (D_a - D_0),$$

where D_a is the density reading of the control (100% undenatured haemoglobin); D_t is the reading at time t; D_0 the reading at completion of the reaction. Therefore, when log H is plotted against time, there results a straight line indicative of the rate of reaction (1).

For the alkali denaturation of the fetal haemoglobin in cord blood the calculation was based on the straight line extrapolated to zero time (3).

Results

The velocity constants for the reactions at different temperatures of sodium hydroxide upon the oxyhaemoglobins of adult and cord blood are plotted in accordance with the Arrhenius equation in Fig. 1, the straight lines being

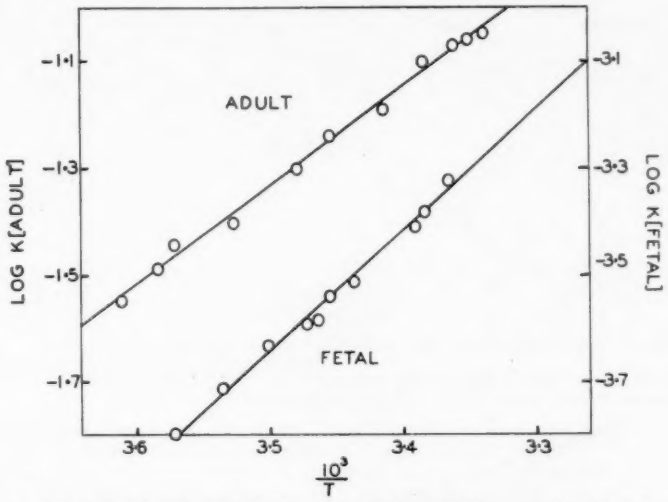

FIG. 1. Alkali denaturation of adult and fetal haemoglobins: relationship of reaction rate to temperature. (K = velocity constant.)

drawn according to the method of least squares. From these, the energy of activation can be calculated, and hence the heat of activation (ΔH^*) and entropy (ΔS^*) (4). For the denaturation of the adult oxyhaemoglobin this gives values for ΔH^* and ΔS^* of 7.9 kcal per mole and -37 entropy units respectively, the corresponding values for the fetal form being 9.7 and -41. The values recorded by Haurowitz *et al.* (2) for the alkali denaturation of human oxyhaemoglobin are $\Delta H^* = 7.6$ kcal per mole and $\Delta S^* = -39$ units.

Figure 2 shows the corresponding Arrhenius relationship for the reactions of urea with adult and cord blood. Figure 3 affords confirmation of the observation that when urea reacts with adult blood there is a change in rate, and shows that the difference between the two rates increases with increasing temperature of reaction. The velocity constant values for adult blood in Fig. 2 represent therefore the initial rate of reaction only.

(2) *The Effect of Variations in Urea Concentration*

All experiments had hitherto been carried out with a urea concentration of approximately 7 M. To ascertain the effect of lower concentrations, the urea solution was suitably diluted with varying amounts of the buffer solution to yield haemoglobin–urea solutions of 6 M, 5 M, 4 M, and 3 M urea respectively. All experiments were carried out as previously described except that the temperature was kept constant at 30° C. Figure 4 shows the results obtained

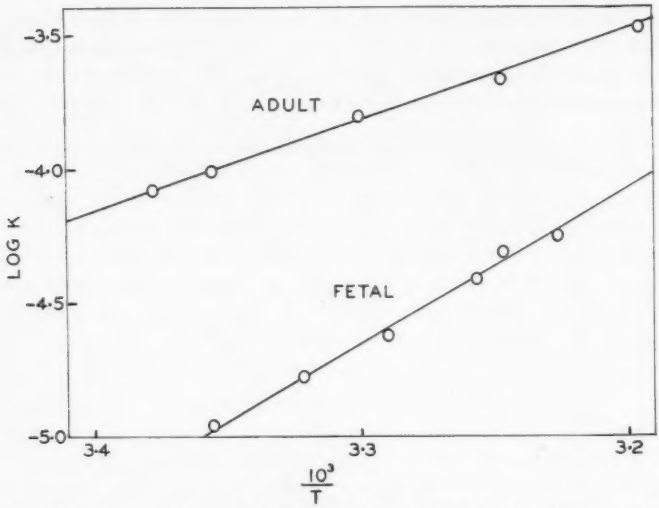

FIG. 2. Urea denaturation of the haemoglobins of adult and cord blood: relationship of reaction rate to temperature. (K = velocity constant.)

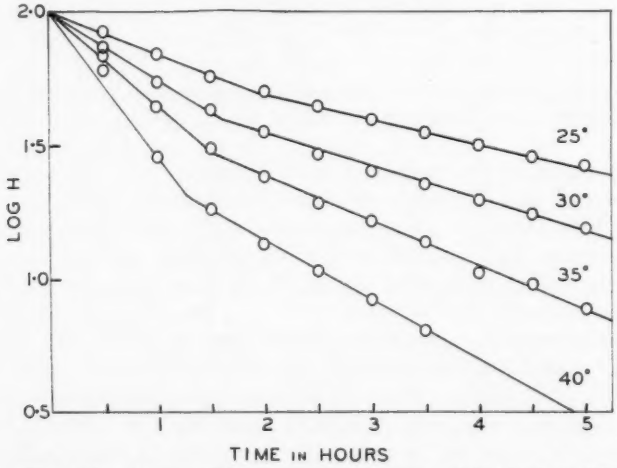

FIG. 3. The action of urea (7 M concentration) upon adult blood at different temperatures. (H = percentage of undenatured haemoglobin.)

with adult blood, the points representing the mean value from a number of experiments, and the straight lines drawn according to the method of least squares. The velocity constants calculated for each concentration of urea are listed in Table I.

A similar series was carried out with cord blood but these are not recorded since the results were substantially the same, although the reactions proceeded at a much slower rate.

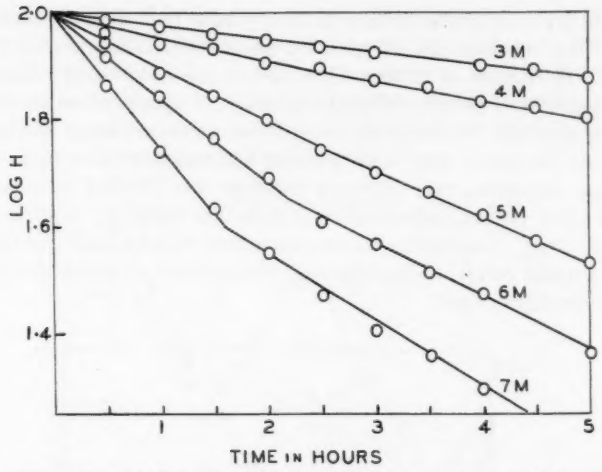

FIG. 4. The action of different concentrations of urea upon adult blood at 30° C. (H = percentage of undenatured haemoglobin.)

TABLE I

Reaction rate constants for the action of varying concentrations of urea upon the haemoglobins of adult blood at 30° C

Urea concentration	K^* (sec^{-1})
7 *M*	1.6×10^{-4}
6 *M*	1.0×10^{-4}
5 *M*	6.4×10^{-5}
4 *M*	2.8×10^{-5}
3 *M*	1.5×10^{-5}

*Initial velocity.

It will be observed that in Fig. 4 the lines representing the rates of reaction with 7 *M* and 6 *M* show distinct breaks; with 5 *M* the break is very slight, while with 4 *M* and 3 *M* the lines appear continuous. The velocity constant for the 4 *M* reaction is 2.8×10^{-5}, while that for 5 *M* is 6.4×10^{-5}. Since the reaction curves with cord blood followed for 5 hours at 30° C showed no signs of a break, and with the highest concentration of urea used (7 *M*) the velocity constant was calculated to be 2.2×10^{-5}, it would appear that it is only when the reaction attains a certain critical velocity (somewhere between 3×10^{-5} and 6×10^{-5}) that a break appears in the reaction curve.

(3) *The Effect of Variations in* pH

In comparing the action on oxyhaemoglobin of urea and other denaturants with that of sodium hydroxide, the pH factor had of necessity to be different; i.e. these reactions had to be carried out at a pH which, although on the alkaline side of neutrality, was still sufficiently lower than the pH of sodium hydroxide solution that there would be no possibility of the result being at least partly due to alkali denaturation. We therefore chose to carry out all these experiments at a pH of 8.5, and the alkali denaturations at pH 11.9.

It was thought that if the action of urea was a true denaturation, then the product of the reaction should give an absorption curve similar to the one obtained by the action of alkali. This did not prove to be the case (1), but it seemed possible that the dissimilarity in shape of these curves could be due to the difference in pH. Accordingly, in a series of experiments adult blood was denatured in the usual way with sodium hydroxide at pH 11.9. When the reaction was complete, the resultant solution was divided into portions and the pH of each portion adjusted to a different value by addition of dilute hydrochloric acid. Absorption curves were then run on each fraction. Figure 5 shows a typical result, indicating that the product of alkali denaturation is affected by changes in pH.

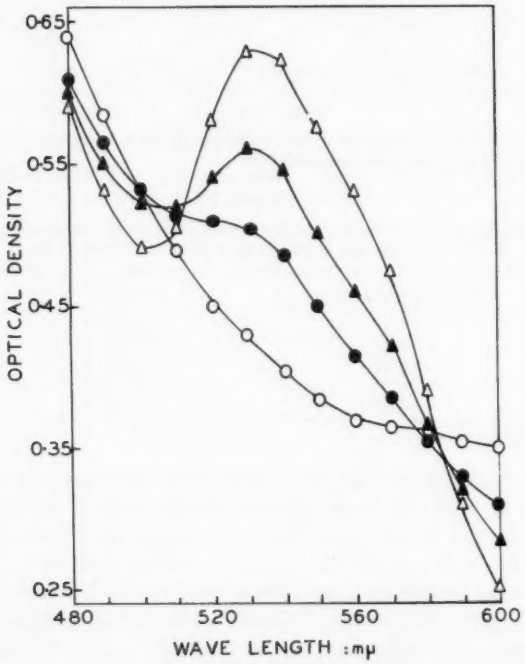

FIG. 5. Product of alkali denaturation of oxyhaemoglobin: absorption curves at different pH values. ○——○ = 11.9; ●——● = 10.5; ▲——▲ = 9.5; △——△ = 8.5.

It will be observed that the curve obtained when the pH was adjusted to 8.5 is of the same shape as the curve reported to result from the action of urea at pH 8.5 (1). It now remained to be seen if the shape of this urea reaction curve could also be modified by carrying out the reaction at a different pH. In this series, a 50% urea solution was made up in the Sørensen buffer as before, and divided into aliquots to each of which varying amounts of normal sodium hydroxide were added drop by drop. The volumes were equalized by

addition of drops of buffer solution, and a measured amount of each aliquot added to the haemoglobin solution in the usual way. The pH was recorded, and the reaction allowed to proceed to completion. It was found that the products of the reaction gave absorption curves whose shapes were affected by the changes in pH in a similar way to those of the product of alkali denaturation. A typical example is shown in Fig. 6.

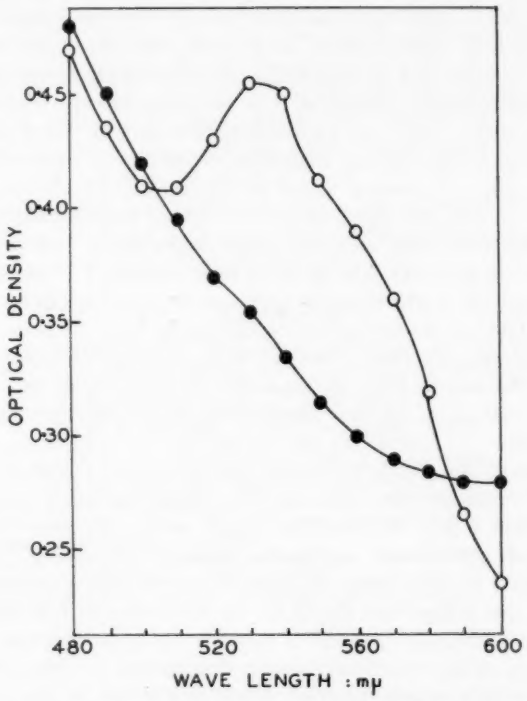

FIG. 6. Product of urea denaturation of oxyhaemoglobin: absorption curves at different pH values. ○——○ = 8.5; ●——● = 10.5.

Discussion

When oxyhaemoglobin is acted on by urea the reaction rate increases with increasing temperature in accordance with the Arrhenius equation. In this respect the urea reaction is similar to that of alkali denaturation as shown in Figs. 1 and 2. From the graphs in Fig. 1 the reaction kinetics for the alkali denaturation of the adult haemoglobin have been calculated, and the figures for the heat of activation and the entropy of the reaction agree reasonably well with those previously reported (2). The corresponding values for the denaturation of the fetal haemoglobin, which have not previously been recorded, show a higher heat of activation with some decrease in entropy. The kinetics of the urea reactions are not reported since the upper graph in Fig. 2

represents only the initial rate of a reaction which shows a change of velocity after a certain period of time (see Fig. 3). The breaks seen in the reaction curves of Fig. 3 would seem to lend support to previous reports that the denaturing action of urea upon the haemoglobins results in the formation of intermediate compounds (5). In particular Banaschak and Jung (6) found that by the action of 5 *M* urea upon horse methaemoglobin at 40° C and pH 7 for 15 minutes the product obtained, when reduced by sodium dithionite, gave an absorption curve with one peak at 557 mμ, whereas if the urea was allowed to react for more than 2 to 3 hours, the corresponding absorption curve after treatment with sodium dithionite showed peaks at 558 and 526 mμ, typical of haemochrome formation. In our experiments the reactions were with oxyhaemoglobin and not methaemoglobin but as the denaturation was invariably found to involve the oxidation of ferrous to ferric iron, it is to be presumed that the end product would be the same.

Apparently no one has previously investigated spectrophotometrically the rates of the reactions with urea and other denaturants, otherwise these very obvious breaks in the curves must have been observed. We have shown (1) that breaks occurred in the reaction curves of oxyhaemoglobin with guanidine, nicotinamide, sodium benzoate, and sodium salicylate. It is unfortunate that we did not investigate the action of sodium dodecyl sulphate also, since Tsushima and Kawai (7) have separated by electrophoresis two spectroscopically identical products of the denaturing action of this substance upon methaemoglobin.

Figure 4 shows the effect of varying the concentration of urea, and again breaks appear in the graphs, but only when these represent a urea concentration greater than 4 *M*. On the other hand, when cord blood was used in a similar series of experiments, no breaks appeared even with 7 *M* urea. The velocity constant of this latter reaction, however, had a calculated value of 2.2×10^{-5} and this is less than the figure for the action of 4 *M* urea upon adult oxyhaemoglobin (2.8×10^{-5}), so it is possible that under these experimental conditions there exists a critical velocity determined by temperature or urea concentration which, when exceeded, leads to a break in the curve.

Steven and Tristram (8) have followed graphically the changes in optical activity and viscosity of ovalbumin in urea solutions of increasing molar concentration and have shown that when the urea reached a concentration of between 4 and 5 *M* the rate of increase in optical activity and in viscosity was markedly accelerated. They consider this to be a critical urea concentration associated with a change in the configuration of the protein molecule after a certain degree of denaturation has occurred.

Our results could perhaps bear a similar explanation since in the reaction of urea with adult oxyhaemoglobin the break in the curve only becomes apparent at a urea concentration of between 4 and 5 *M*. It is true that this "critical urea concentration" apparently does not hold for the reaction with cord blood, but this may be merely one more of the many points of difference between adult and fetal haemoglobins (5, 9).

Finally, the evidence presented in Figs. 5 and 6 indicates that the actions of urea and sodium hydroxide upon oxyhaemoglobin yield a spectroscopically similar product, the shape of the absorption curve being influenced by the pH. The product of the urea reaction would therefore appear to be the substance which is known variously as kathaemoglobin or parahaematin or denatured globin haemichrome (10, 11), a substance giving a characteristic spectrum with a weak band at 558 mμ and a stronger one at 530 mμ. This product apparently is perfectly stable at pH 8.5, but as the pH is increased, the absorption bands are eliminated with the formation of the substance we have referred to as alkaline globin heamatin (3), the end result of alkaline denaturation. It has been known for some time (12) that the kathaemoglobin bands could be produced by neutralizing the alkali denaturation product with carbon dioxide or acetic acid. Figure 5 shows that the characteristic peaks can appear while the solution is still definitely alkaline.

It would therefore appear from the results now reported that the action of urea upon oxyhaemoglobin is a true denaturation, and forms an end product similar to that produced by alkali denaturation.

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EFFECTIVENESS OF YEAST, VITAMIN B_{12}, AND ALTERNATIVE TREATMENTS ON SURVIVAL OF INDIAN CARP DURING THE EARLY PERIOD OF LIFE[1]

B. C. Das

Abstract

Standardized preparations of yeast and vitamin B_{12}, and some alternative treatments, were administered to Indian fresh-water carp (*Catla catla*, *Labeo rohita*, *Cirrhina mrigala*) during the first 2 weeks of life following hatching, to determine their effect on survival.

A randomized block design was employed to set up 54 treatment groups, with 9 replications for each of 6 treatments. The daily treatments per 9 liters of water were: 0.324 g yeast; 25 μg crystalline vitamin B_{12}; 10 ml of ruminant stomach extract; 1 μg cobalt nitrate; 10 ml ruminant stomach extract with 1 μg cobalt nitrate; and an untreated control. Significant differences ($P < .01$) were observed between the proportion of carp surviving in the control and in treatments with yeast, vitamin B_{12}, ruminant stomach extract, and ruminant stomach extract with cobalt nitrate. Yeast was the most effective treatment, with 89% surviving; with ruminant stomach extract with cobalt nitrate 84% survived; with ruminant stomach extract alone 83% survived; with vitamin B_{12} 75% survived; while 65% survived in the untreated control. The results indicate that some components of the vitamin B complex, including B_{12}, are effective in increasing survival during the early period of life. Alternative treatments utilizing ruminant stomach extract, possibly containing these components, are also adequate for increasing survival.

Introduction

In many species it has been shown that the early phase of life is characterized by a low rate of survival. Although it is known that the rate of survival increases with age up to maturity, the patterns of survival are less widely established. Day-by-day observation of survival in relation to age has been carried out in this laboratory on Indian fresh-water carp, particularly for the major species *Catla catla*, *Cirrhina mrigala*, and *Labeo rohita*, and for the minor species *Labeo bata*, *Cirrhina reba*, and *Crossocheilus latia* (1, 2, 3, 4). The pattern of survival which emerges from these observations is one of very low survival for the first week of life after hatching, followed by a steady increase with age.

It has also been observed that survival during the early period of life in Indian carp can be significantly enhanced by treatment with minute quantities of vitamin B complex and B_{12}. While the growth-promoting and deficiency-correcting functions of these vitamins are well established (5), their importance for survival during the postembryonic stage of life, particularly in fish, has not been widely demonstrated. In other species, it has been suggested that susceptibility to various adverse factors may be attributed to an imbalance of metabolic processes which is associated with the rapid growth characteristic of the early period of life. The results with Indian carp suggest that certain factors in the B complex may play an important role in the balance of meta-

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bolic processes during this period but with increasing age, they become less effective.

The ability of intestinal organisms to synthesize such components of the B complex as folic acid, nicotinic acid, pyridoxine, thiamine, and riboflavin has also been noted (5). The digestive tract of the ruminant has also been shown to be capable of synthesizing vitamin B_{12} in the presence of cobalt (10). Whether or not these natural sources would yield substances capable of enhancing survival in fish to the degree attained by known sources of B complex and B_{12} has not been reported.

The present experiment was conducted to determine the effect of treatments, which could be considered as alternative to standardized preparations of B complex and B_{12}, on the pattern of survival during the early period of life following hatching in Indian fresh-water carp.

Statistical Design of the Experiment

Fifty-four experimental units were allocated to six treatments and nine blocks according to a randomized block design. Within each block, the treatments were randomly ordered. Each treatment was replicated nine times, once in each of the nine blocks. The six treatments, their symbols, and dosages were as follows:

Symbol	Treatment	Dosage per 9 liters
A	Control	—
B	Cobalt nitrate	1 part per million (1 μg)
C	Ruminant stomach extract	10 ml
D	Yeast	1 tablet
E	Vitamin B_{12}	25 μg
F	Cobalt nitrate and ruminant stomach extract	1 μg and 10 ml

Materials and Methods

One-day-old carp of the major species (*Catla catla*, *Cirrhina mrigala*, *Labeo rohita*) were obtained from the same lot. As the exact enumeration of the carp was not feasible due to their minute size, they were allocated to the experimental units according to a sampling procedure as follows: 1 teaspoon of carp was assigned randomly to each experimental unit. There were 54 experimental units, each consisting of an earthen bowl 16 in. in diameter, containing 9 liters of water, and covered with screening. To prevent accumulation of waste products, the water of each unit was changed every 24 hours by a siphoning technique which prevented loss of carp. Fresh pond water was obtained from the same source. The water was maintained at 9 liters for each experimental unit. Throughout the experimental period, water temperatures did not exceed 28.5° C and were not less than 25.5° C; similarly, pH ranged from 8.7 to 7.9, and the room temperature from 34.5° C during the day to 22.9° C at night. Fluorescent tube lights were arranged to ensure uniform lighting throughout the laboratory. Fans were arranged to keep the air in constant circulation and to maintain a constant temperature throughout the laboratory. The carp were fed live *Daphnia* which were cultivated in a richly fertilized

artificial pond, following the recommended cultural practices (6) for carp fry culture. Ten milliliters of a solution with 5 parts *Daphnia* by volume to 95 parts water was given daily to each experimental unit. As daily checks revealed that some *Daphnia* always remained, this ration was considered sufficient.

The dead carp were completely enumerated each day at the same time to obtain the experimental counts. After a centrifugal current was induced in the water, the dead carp settled in the bottom of the experimental unit and were withdrawn with a glass pipette, placed on blotting paper, and counted. A fresh pipette was used for each experimental unit each day. At the end of the experiment, the number of live carp (of an average length of 1 cm) was counted. The initial number of carp was reconstructed by adding the total number of dead carp to the number alive at the end of the experiment.

The daily dosage per 9 liters of water for the different treatments was as follows:

1. Each experimental unit belonging to treatments B and F received 1 μg of cobalt nitrate daily. The molarity of the cobalt nitrate was 3.43×10^{-6}. Total impurities present did not exceed 0.045%.
2. Each experimental unit in treatments C and F was given 10 ml of ruminant stomach extract daily. The extract consisted of the juice expressed from the entire contents of the four stomachs of a freshly slaughtered goat. No further water was added.
3. Each experimental unit in treatment D received daily one tablet which contained 0.324 g yeast (British Drug Houses Ltd.).
4. Each experimental unit in treatment E was given one 25-μg tablet of crystalline vitamin B_{12} (British Drug Houses Ltd.) daily.

Results

Table I gives the initial and final numbers of carp in each treatment. For each replication, the proportion surviving was computed by dividing the final number by the initial number. The average of these values gives the mean final proportion surviving, and the standard deviation indicates the variability. Table I presents the differences between the control mean and treatment means, and the differences between the mean for B_{12} and other treatment means. The comparisons show that survival in treatments C, D, E, and F is significantly greater than in the control, and that survival in treatments D and F is higher than in treatment E with B_{12}. The significance of the differences was evaluated by the critical differences given in Table II.

A two-way analysis of variance was carried out on the final proportions surviving to determine whether the treatments and blocks differed significantly (9). The analysis showed that the treatments differed significantly at the 1% level of confidence, but that the blocks did not differ significantly. Using the error mean square of the analysis of variance, the critical differences required for the difference between any two treatments to be significant at the 1% and 5% levels were computed. The analysis of variance summary table is given in Table II.

TABLE I

Initial and final numbers, and means, standard deviations, and comparisons of final proportion surviving

	Treatment					
	A	B	C	D	E	F
	Control	Cobalt nitrate	Ruminant stomach extract	Yeast	Vitamin B_{12}	Cobalt nitrate and ruminant stomach extract
Initial number of fish	3168	2501	3927	4582	3443	3215
Final number of fish	2017	1648	3219	4093	2614	2746
Mean final proportion	.6478	.6709	.8263	.8929	.7524	.8434
Difference of mean from control		.0231	.1785**	.2451**	.1046*	.1956**
Difference of mean from B_{12}			.0739	.1405**		.0910*
Standard deviation of final proportion	.0841	.1110	.0653	.0123	.1290	.0526

*$P < .05$.
**$P < .01$.

TABLE II

Analysis of variance summary table for final proportion surviving

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between treatments	.4381	5	.0876	10.4286**
Between blocks	.0530	8	.0066	.7857
Error	.3373	40	.0084	
Total	.8284	53		

**$P < .01$.
NOTE:
5% critical difference $= 2.010 \times \sqrt{.0084} \times \sqrt{(1/9+1/9)} = .0870$.
1% critical difference $= 2.681 \times \sqrt{.0084} \times \sqrt{(1/9+1/9)} = .1161$.

The trend of survival is shown in Table III, which gives the mean proportion surviving for each day of the experimental period by treatments.

TABLE III

Mean proportion surviving for each day showing the trend of survival

	Treatment					
	A	B	C	D	E	F
Day of experiment	Control	Cobalt nitrate	Ruminant stomach extract	Yeast	Vitamin B_{12}	Cobalt nitrate and ruminant stomach extract
1	.9294	.9163	.9374	.9747	.9536	.9533
2	.8264	.8026	.8888	.9186	.8691	.9066
3	.7007	.7067	.8589	.9021	.7999	.8750
4	.6752	.6923	.8492	.8999	.7817	.8672
5	.6546	.6783	.8466	.8974	.7721	.8567
6	.6531	.6764	.8451	.8951	.7687	.8537
7	.6515	.6758	.8422	.8947	.7654	.8520
8	.6505	.6748	.8373	.8939	.7632	.8499
9	.6496	.6716	.8303	.8933	.7602	.8474
10	.6481	.6713	.8271	.8930	.7537	.8448
11	.6478	.6709	.8263	.8929	.7524	.8434

Discussion

The data presented in Table I show that, while 65% survive without treatment, 75% survive with the addition of B_{12}, and 89% survive with yeast treatment. The analysis of variance (Table II) indicates that the differences between treatments are statistically significant ($P < .01$), and using the critical difference, the significance of the difference between means has been evaluated. Both B_{12} and yeast are found to enhance survival significantly in comparison with the control. As the control fish were fed a diet of *Daphnia* which had been cultivated in a richly fertilized artificial pond, it would not be expected that they were deficient in the essential nutrients. The survival of 65% in the control also suggests that the carp were not suffering from malnutrition, in which case a much lower percentage would survive. The vitamin and yeast treatments were effective even in minute quantities. While 25μg of B_{12} was given daily to 9 liters of water, the yeast preparation provided 0.324 g dehydrated Brewer's yeast, 0.06 mg thiamine hydrochloride, 0.03 mg riboflavin, and 0.15 mg niacin for 9 liters of water daily. While the B_{12} was from a standardized source, the yeast provided a natural source of B complex (5). Comparison of the survival with these treatments with that obtained by treatment with B complex of known composition is provided by another experiment carried out concurrently. In that experiment, the mean proportion surviving was 0.7733, and the standard deviation was 0.0127. The composition of the B complex was: 10 mg thiamine hydrochloride, 3 mg riboflavin, 2 mg pyridoxine hydrochloride, 15 mg nicotinic acid amide, and 3 mg calcium pantothenate. These data indicate that B complex of known composition can effectively enhance survival of fish at this stage of life, though not as effectively as yeast extract in the present experiment.

In practical fishery or livestock work, the cost and availability of vitamins or supplement rations must be considered in addition to their effectiveness. Previous experiments in this laboratory were directed toward utilization of materials which might be expected to yield effects similar to those of standard preparations of the B vitamins. As the ruminant digestive tract is known to contain microorganisms which synthesize vitamin B_{12} (10), it was postulated that extract of the ruminant's several stomachs might produce the desired effect. A locally available ruminant, the goat, was chosen for this investigation. In earlier experiments, a diluted extract yielded beneficial results when it was given along with cobalt nitrate (1). It was hypothesized that, in the presence of cobalt, the microorganisms in the digestive tract of the ruminant were able to synthesize B_{12} (10), which in turn enhanced the survival of the treated carp. A more concentrated extract, used in this experiment, resulted in 83% survival alone, and 84% survival when mixed with cobalt nitrate. Treatment with the extract alone is significantly better than the control, but not significantly different from yeast or B_{12}. Treatment with ruminant stomach extract with cobalt nitrate was significantly better than B_{12} in increasing survival, but did not differ significantly from treatment with yeast. These results suggest that intestinal organisms are capable of producing some components of the B complex, possibly including B_{12}, in sufficiently large quan-

tities to reduce mortality during the early period of life in Indian fresh-water carp.

During the first week of life following hatching, the likelihood of Indian carp dying is greater than it is during the second week. The survival trend is illustrated by the data in Table III, which show that, while the proportion alive decreases rapidly during the first few days, it decreases at a much slower rate during the following days. The effectiveness of the treatments in altering this pattern of survival is apparent by the third day of the experiment. While the proportion surviving dropped from 0.9294 to 0.7007 on the third day in the control, for carp under yeast treatment the corresponding drop was from 0.9747 to 0.9021. Decrease in survival of a similar magnitude to that of yeast was observed for treatment with ruminant stomach extract, with or without cobalt nitrate. By the end of the experimental period, by which time the carp were in their second week of life, less than 1% died each day, regardless of experimental treatment. These results suggest that the very young carp, in the phase immediately following hatching, are more sensitive to treatment with some component(s) of the B complex, from either standardized or natural sources. Furthermore, while the growth-promoting and disease-preventing functions of these vitamins are well known, the present data suggest that survival itself, in fish during the postembryonic stage of life, may be affected by the vitamins present.

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THE IN VITRO INCORPORATION OF PHOSPHORUS-P^{32} INTO TOBACCO MOSAIC VIRUS[1]

P. M. TOWNSLEY

Abstract

Tobacco mosaic virus can be labelled with P^{32} during homogenization of the diseased tissue with a soluble, stable, unknown phosphate compound. The label remains associated with the virus during purification and can be isolated from the virus by incubation in potassium hydroxide. The labelled phosphate compounds released are mainly adenylic, cytidylic, uridylic, and guanylic acids. The addition of *p*-fluoro-DL-phenylalanine and 2-thiouracil reduces the amount of P^{32} found in the virus. Chloramphenicol does not inhibit P^{32} incorporation into TMV.

The study of the synthesis of plant viruses is complicated by the fact that to date no one has succeeded in synthesizing a virus outside of the living cell. For while it is true that in vitro recombination of virus protein and virus nucleic acid has been achieved (1), it is obvious that this can at best represent the final stage of the process, if indeed it is at all typical of what occurs in the plant cell (2).

A preparation of phosphate-labelled tobacco mosaic virus (TMV) can be obtained by the isolation of the virus from the extracts of labelled leaves (3, 4, 5). It is the purpose of this paper to show that at least a portion of the labelled virus nucleic acid may be derived from phosphate-containing compounds present during the homogenization of the diseased plant tissue. It will also be shown that the virus can be labelled in vitro by the addition of a labelled phosphate fraction derived from healthy plant tissue to cell-free preparations of TMV.

General Methods

Both healthy and TMV diseased tobacco plants, *Nicotiana tabacum* L. var. Haronova, were grown under greenhouse conditions. Only lower leaves, about 14 in. long, were used in the experiments. In experiments involving the early stages of TMV infection, the leaves of healthy plants were uniformly inoculated with a diseased leaf homogenate. The debris was washed off the inoculated leaves and the leaves were harvested 40 to 48 hours later.

Leaf disks, made with a ¼-in. cork borer, were cut from the leaves and placed under fluorescent light in Hoagland's solution containing radioactive orthophosphate (4). After 4 hours' incubation the disks were blotted dry and homogenized for 1 minute at 3° C in a Potter homogenizer using a 1-ml conical centrifuge tube. The homogenates were centrifuged for 1 minute at $2000 \times g$ and the supernatant was fractionated by agar gel electrophoresis. The P^{32} was located by autoradiography (4).

[1]Manuscript received December 3, 1959.
Contribution No. 14, Research Station, Canada Agriculture, c/o The University of British Columbia, Vancouver 8, British Columbia.

Results

Tobacco mosaic virus became labelled within a few hours of immersing the leaf petioles (4) or leaf disks in radioactive orthophosphate solution (Fig. 1). The size of the diseased leaf, leaf disk, or segment incubated in the P^{32} solution

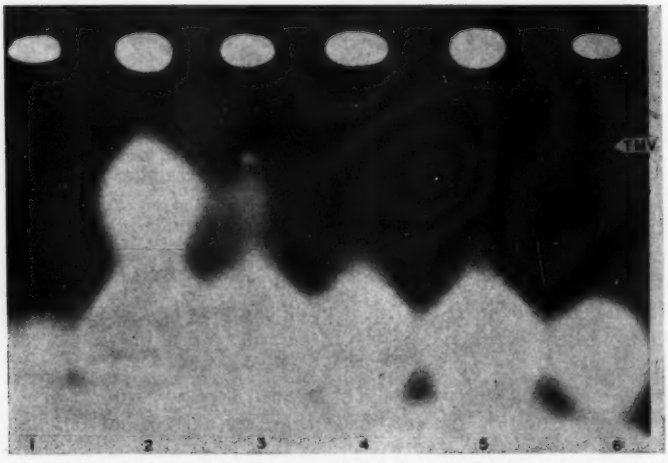

FIG. 1. An autoradiogram showing that P^{32} labelling of TMV in diseased leaves is independent of the size of the leaf fragment.

Leaves from a plant several months after inoculation were prepared as below and approximately equal amounts of leaf surface area were placed in 1.6 ml of Hoagland's solution containing 7 μc of P^{32} orthophosphate for 4 hours. Then the samples were homogenized, centrifuged at $2000 \times g$, and the supernatant fractionated on agar gel electrophoresis.

(1) Homogenate; (2) leaf pieces $\frac{1}{16} \times \frac{1}{8}$ in.; (3) leaf pieces $\frac{1}{4} \times \frac{3}{4}$ in.; (4) leaf pieces $\frac{1}{2} \times \frac{3}{4}$ in.; (5) leaf pieces $\frac{1}{2} \times \frac{3}{4}$ in. including large veins; (6) whole leaf 5 in. long immersed by the petiole.

did not appear to be critical to the ready formation of labelled virus. When the diseased tissue was homogenized before the addition of P^{32} orthophosphate, labelled virus was not formed; in fact, the orthophosphate did not appear to be readily metabolized under these conditions. If the labelled orthophosphate of Hoagland's solution was replaced by an equivalent amount of uridine-5′-phosphate P^{32}, the virus isolated from the leaf disks was not labelled as rapidly as with orthophosphate. Since there was an excess of free labelled uridylic acid present during homogenization of the tissue, the possible adsorption of this labelled nucleotide would not account for all the labelled virus prepared by using orthophosphate.*

Considering that plants have a mechanism for the synthesis of nucleic acid and nucleoprotein, it would seem quite likely that at least a part of this mechanism could be utilized to provide a source of phosphate compounds required for viral nucleic acid synthesis. Therefore, an experiment was carried out to determine whether healthy tobacco leaf disks labelled with P^{32} would

*P^{32} labelled uridine-5′-phosphate was supplied through the kindness of Dr. G. M. Tener, B.C. Research Council, Vancouver 8, B.C.

transfer some of this label to the virus during the homogenization with the diseased tissue and the subsequent isolation procedure. The virus isolated was radioactive (Fig. 2). To reduce the complexity of this transfer of phosphate

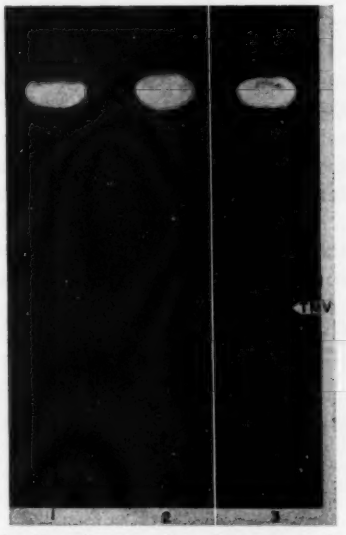

FIG. 2. The transfer of radioactive phosphate derived from healthy leaf tissue to TMV during leaf homogenization.

Twenty-five healthy leaf disks were floated on 1.6 ml of P^{32} orthophosphate (6 μc) in Hoagland's solution for 4 hours. The disks were blotted dry and homogenized with (1) 0.15 ml of TMV protein (17 mg, pH 6.5); (2) 25 diseased leaf disks; (3) the healthy labelled homogenate permitted to stand at 35° C for 5 minutes before homogenization with 25 diseased leaf disks. After electrophoresis the agar gel was washed with 70% ethanol.

to TMV, a labelled supernatant preparation was prepared by centrifugation of the healthy labelled leaf disk homogenate at 20,000$\times g$ for 1 minute. The supernatant was added to diseased leaf disks immediately prior to their homogenization. Again the virus was labelled.

Further experiments indicated that the phosphate compounds present in healthy supernatants, which were transferred to TMV, were stable to incubation for at least 1 hour at 0° C and to 2 minutes at 80° C. Also the compounds were capable of being separated from the healthy supernatant by electrodialysis and were soluble in 70% alcohol.

Labelled TMV was also prepared by adding the clarified healthy phosphate source to a diseased leaf homogenate. However, the amount of radioactivity transferred to TMV was greatly reduced, as shown by visual observation of the autoradiograms, in comparison to the radioactivity found in TMV if the phosphate source was added just prior to homogenization.

At this point in the investigation, it was thought that the virus protein which was found in apparently free form in virus infections (6) and sometimes referred to as soluble antigen may serve as a site for polymerization of labelled

nucleic acid. However, the supernatant from an ultracentrifuged TMV extract of a diseased leaf, which contained soluble antigen, did not form labelled TMV when homogenized with healthy labelled leaf disks. A similar negative result was obtained when the virus protein, prepared by the method of Fraenkel-Conrat (1957), was homogenized with healthy labelled leaf disks (Fig. 2).

When crude virus preparations, prepared by centrifugation of a diseased homogenate at 20,000×*g* or by a single differential centrifugation at 2000×*g* and 78,000×*g* respectively, were homogenized with labelled healthy leaf disks, the TMV became labelled. However, the amount of label transferred to TMV was much less than when diseased leaf disks were homogenized with labelled healthy leaf disks.

Two experiments were performed to demonstrate that nucleic acid was being labelled. Tobacco mosaic virus, labelled by homogenizing healthy leaf disks containing P^{32} with TMV diseased leaf disks, was prepared with carrier non-labelled TMV by four alternate centrifugations of 2000×*g* and 78,000×*g*. After the first ultracentrifugation the virus pellet was incubated for 18 hours at room temperature in 0.1 *M* sodium citrate at pH 6.0 (8) to remove contaminating normal nucleoprotein. The virus pellet was washed with 70% ethanol, alcohol–ether, 0.2 *N* perchloric acid, and 70% ethanol (9). The nucleotides were isolated by treating the washed pellet with 0.33 *N* KOH for 18 hours at 37° C (10, 11). The hydrolyzate was neutralized with 9 *N* perchloric acid and the nucleotides were separated by paper electrophoresis at pH 3.5 in 0.05 *M* ammonium formate buffer. Six radioactive bands were observed corresponding to the four nucleotides of TMV and to a small amount of radioactivity due to inorganic phosphate, and to material remaining at the point of application. Since orthophosphate as such does not label the virus (3) it must be assumed that the radioactive material transferred to the virus was probably composed of nucleotides. These were probably not adsorbed nucleotides because of the insignificant adsorption with labelled uridylic acid and the washing procedure used before alkaline hydrolysis of the nucleoprotein. Also, they were not likely to be derived from healthy adsorbed nucleoprotein because this nucleoprotein is removed by incubation of the virus in citrate solution.

Labelled virus was also prepared by homogenizing healthy labelled tissue with TMV diseased tissue, and was isolated from agar gel after electrophoresis. Both the virus band and a corresponding area in the agar gel from healthy labelled tissue were cut out and washed successively for 24 hours with 70% ethanol containing 0.015 *M*, pH 5.0, acetate buffer, followed by 70% ethanol and ether. The radioactivity of the washed gel was eluted with 0.33 *N* KOH for 18 hours at 37° C on a shaker. The eluate, which was found to contain approximately 90% of the radioactivity, was brought to pH 2.5 with perchloric acid and the resultant precipitate removed by centrifugation. The supernatant was adsorbed onto a 2.5×1.0 cm Norite A activated charcoal column (12). The column was washed with water and the nucleotides eluted with 50% ethanol containing 2% NH_4OH. The eluates were concentrated to dryness

under vacuum and redissolved for nucleotide determination by paper chromatography (13). The developing solvent was isobutyric acid – *N* ammonium hydroxide – 0.1 *M* ethylenediaminetetracetic acid disodium salt. Almost all of the radioactivity eluted from the charcoal was found as adenylic, cytidylic, and uridylic acids. Guanylic acid was present in only a small amount due to difficulty in eluting this compound from Norite A. The total radioactivity of the nucleotides isolated from the TMV-containing agar gel greatly exceeded the nucleotide radioactivity found in a similar area in the 'healthy' sample.

Nothing is known of the method by which phosphate becomes incorporated into TMV, but some experiments with an amino acid analogue present an interesting consideration. It was noted that when *p*-fluoro-DL-phenylalanine* (FPA) was added to radioactive nutrient solution in which 40- to 48-hour infected leaf disks were incubated, radioactivity in the virus was inhibited greatly (Fig. 3). When a similar experiment was done using leaf tissue that

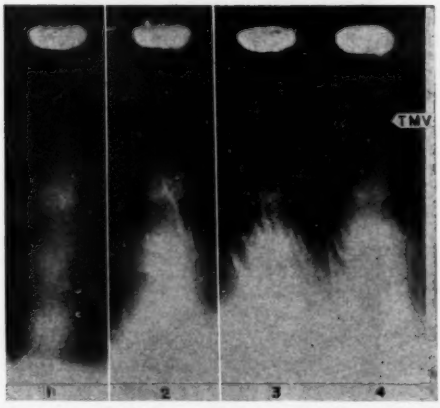

FIG. 3. The incorporation of P^{32} into a 40-hour infection of TMV in the presence of *p*-fluoro-DL-phenylalanine and 2-thiouracil. (1) Forty healthy leaf disks; (2) 40 TMV leaf disks; (3) 40 TMV leaf disks + 0.71 mg FPA/ml; (4) 40 TMV leaf disks + 1.7 mg 2-thiouracil/ml. All nutrient solutions contained 3 μc/ml of P^{32} orthophosphate and were adjusted to pH 7.0 before the addition to the leaf disks. After an incubation of 4 hours the leaf disks were homogenized and the extracts analyzed by electrophoresis.

had been infected for several months, the radioactivity, by visual comparison of the autoradiograms, in the virus was decreased approximately 50%. Similar experiments with DL-phenylalanine did not show inhibition. It remains to be elucidated whether the decrease in radioactivity in the TMV band in the presence of FPA resulted from decreased general cell metabolism or from inhibition of a virus-synthesizing mechanism. Nevertheless, when a non-radioactive diseased leaf from a 3-month-old TMV infected plant was homogenized with radioactive 44-hour diseased leaf disks that had been incubating in FPA, an increase in radioactivity was observed in the virus (Fig. 4). Undoubtedly there was radioactive material present in the FPA, 44-hour infected

*p-Fluoro-DL-phenylalanine supplied by California Foundation for Biological Research, 3408 Fowler St., Los Angeles, California.

leaf disks which apparently did not label TMV *in situ* but did form labelled TMV if older infected TMV tissue were added during the homogenation.

FIG. 4. The observed counteraction of *p*-fluoro-DL-phenylalanine inhibition of P^{32} incorporation into TMV. (1) Forty TMV leaf disks 44 hours after inoculation with TMV+ Hoagland's solution containing 7 μc P^{32} orthophosphate; (2) similar to (1) + 1.0 mg FPA/ml Hoagland's solution; (3) similar to (2). After 4 hours' incubation the samples were homogenized as follows: (1) with 40 non-labelled TMV leaf disks from leaves 48 hours after inoculation with TMV; (2) same as (1); (3) with 40 non-labelled TMV leaf disks from plants 3 months after inoculation with TMV. After electrophoretic separation of the samples, the dried gel was dialyzed against 70% alcohol for 8 hours (4).

Webster (14) observed that when chloramphenicol was added to plant ribonucleoprotein particles, protein synthesis was inhibited. If it is assumed that this inhibitor also prevents TMV protein synthesis, then it is of interest to determine whether the TMV in diseased leaf disks placed in chloramphenicol and labelled phosphate accepts the label. Adding chloramphenicol in concentrations varying from 2.5 to 400 μg per milliliter of nutrient solution to diseased leaves ½ hour before addition of P^{32} did not prevent labelling of the virus in whole leaves (Fig. 5). However, it brought about an increase in radioactivity in a zone within the agar gel corresponding to free high polymer nucleic acid in both healthy and diseased tissue. Occasionally, it was noted that chloram-

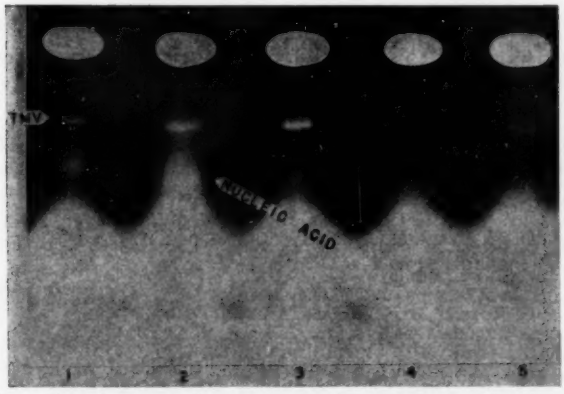

FIG. 5. The action of chloramphenicol on the distribution of radioactive phosphate in TMV diseased leaves.

Excised TMV leaves were immersed in Hoagland's solution containing 200, 100, 50, 5, 0 μg per ml respectively of chloramphenicol for ½ hour (4). Radioactive orthophosphate solution was added and after an incubation period of 6½ hours the tissues were ground in a mortar and pestle and the extract analyzed by electrophoresis in agar gel.

phenicol caused an increase of radioactivity of the diseased samples in the origin position of the electrophoresis over that of the healthy preparation similarly treated. Furthermore, when 2-thiouracil, a nucleic acid analogue, was added to the nutrient solution, the P^{32} activity in the TMV zone decreased and the activity found in the origin position increased (Fig. 3).

Discussion

Although the biosynthesis of tobacco mosaic virus is undoubtedly a very complicated reaction, it would appear possible from these experiments that at least a part of the synthetic reaction may be carried out in broken cell preparations. A soluble, stable phosphate compound, derived from healthy tissue, is incorporated into TMV.

Examination of the preparations containing labelled virus by agar gel electrophoresis has failed to locate the accumulation of any characteristic labelled virus particle other than a single radioactive band. Ultracentrifugation of virus preparations and treatments such as incubation in citrate solutions causes a number of virus bands to appear on agar gels, but these are thought to arise as a result of the treatment on the whole virus (4). There are no apparent bands representing possible steps in the synthesis of the virus polymer.

The inhibition of phosphate incorporation into TMV by FPA is interesting since it is difficult to visualize how an amino acid analogue could interfere with viral nucleic acid synthesis. This inhibition would suggest that there is a relationship between amino acids and the synthesis of viral nucleic acid. On the other hand, if one assumes that virus protein synthesis is inhibited by chloramphenicol the incorporation of phosphate in the virus would be independent of this inhibition.

Acknowledgment

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STUDIES ON THE NUTRITIONAL VALUE OF DEFATTED FISH FLOUR[1]

A. B. MORRISON AND J. A. CAMPBELL

Abstract

In studies on the nutritional value of defatted fish flour, groups of male weanling rats were given otherwise adequate diets containing 7%, 10%, or 15% protein supplied by fish flour or casein. After 4 weeks, protein efficiency ratio (P.E.R.) values found for 7% and 10% fish flour were significantly higher than those for comparable levels of casein, but similar values were found for both proteins at the 15% level. The kidneys of the animals given diets containing 15% protein were significantly heavier than those of the animals fed the other diets, but level or source of dietary protein had no significant effects on liver and adrenal weights, or liver lipid levels.

In further studies, fish flour was added to whole wheat flour or to white bread prior to baking, and the resulting mixtures were fed to growing male rats at the 10% protein level. Addition of 2, 4, or 6 lb of fish flour per 100 lb of whole wheat flour increased the P.E.R., in a stepwise manner, from 1.49 to 2.80 but had no effect on organ weights or liver lipid levels. Addition of 10% fish flour to white bread made with or without 4.2% milk solids increased the P.E.R. values by 82% and 198% respectively, and significantly decreased liver lipid levels. The P.E.R. values for bread and flour diets were found to be a direct function of the lysine content of the protein.

It was concluded that defatted fish flour is an excellent source of high quality protein, and should be of particular value in supplementing diets deficient in lysine.

Introduction

The well-known high nutritional value of fish protein (1) has prompted studies on the use of fish flour to supplement low-protein diets in underdeveloped countries. Autret and Van Veen (2) described experiments conducted in various parts of the world by the Food and Agriculture Organization of the United Nations, in which edible fish flour was incorporated into the diets of infants and children. In almost all cases, foods containing as much as 10% fish flour were well accepted. Sure (3, 4) found that the addition of small amounts of defatted fish flour to the diet of growing rats markedly increased the protein efficiency ratios (P.E.R.) found with various cereal grains. The present experiments were conducted with growing rats to provide information on the effects of dietary protein level on the P.E.R. of fish flour and to study further the effects of fish flour on the nutritional value of whole wheat flour and white bread. Data on P.E.R., organ weights, liver lipid levels, and lysine content of the protein were obtained.

Experimental

In each of the three experiments reported herein, male weanling rats of the Wistar strain were individually housed in screen-bottom cages kept in an air-conditioned room maintained at 74° to 76° F. Food and water were supplied ad libitum, records were kept of the amount of food consumed by each rat, and the animals were individually weighed at weekly intervals. All experi-

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

ments were 28 days in duration, at the end of which time the animals were killed by ether anesthesia, exsanguinated, and the livers, kidneys, and adrenal glands were removed and weighed. Liver lipid levels were determined by the method of Folch *et al.* (5) and were expressed on a wet-weight basis. In addition, liver cholesterol levels were determined on the animals in experiment 3, by a modification of the method of Abell *et al.* (6).

The basal protein-free diet used in the experiment was that of Chapman *et al.* (7), which contained 80% corn starch, 10% corn oil, 5% non-nutritive cellulose, 4% salts, and 1% vitamin mixture. The protein-containing materials were added to this diet at the expense of cornstarch, to give the desired protein levels. Dietary fat levels were kept constant at 10% by adjustment of the amount of corn oil added.

Experiment 1 was conducted to determine the protein efficiency ratio of fish flour fed at various protein levels. Comparable groups of rats received diets containing 7%, 10%, or 15% protein (N×6.25), supplied by fish flour or casein. The fish flour used in this and subsequent experiments was a defatted sample which contained 90% protein (N×6.25), 5.4% moisture, and 6.4% ash, and was made from codfish muscle.

The effect of small amounts of fish flour on the nutritional value of whole wheat flour was tested in experiment 2. Fish flour was added to whole wheat flour at the level of 2, 4, or 6 lb per 100 lb of the wheat flour, and the resulting mixtures were incorporated into the protein-free basal diet to give a dietary protein level of 10% (N×6.25).

In experiment 3, four samples of enriched white bread were made according to a commonly used bread recipe. Two of the samples contained 4.2% milk solids, whereas the other two samples were made with water. Prior to baking, 10% fish flour was added to one of the samples made with milk and to one of those made with water, at the expense of white flour. After baking, the bread samples were sliced, dried at room temperature, ground, and incorporated into the basal diet at the 10% protein level (N×6.25).

The lysine content of the flour and bread diets in experiments 2 and 3 was determined by the method of Steele *et al.* (8), using *L. mesenteroides* P-60 as the assay organism. Acid hydrolyzates were prepared by refluxing the samples with 20% (v/v) hydrochloric acid for 8 hours. The samples were adjusted to pH 4 to remove interfering materials (9) before being neutralized and made up to volume.

The data were subjected to appropriate statistical analyses by methods outlined by Snedecor (10).

Results and Discussion

Effect of Protein Level on P.E.R.

The results of experiment 1 are summarized in Table I. The weight gains and P.E.R. values found with 7% or 10% fish protein (diets 4, 5) were significantly higher ($P<0.01$) than those found with comparable levels of casein (diets 1, 2). However, the values found with 15% protein supplied by casein were similar to those found with fish flour. The P.E.R. values found with

TABLE I

Nutritional value of casein or defatted fish flour fed at three different levels to growing male rats for 4 weeks (experiment 1)

	Casein			Fish flour		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Diet protein, %	7	10	15	7	10	15
Initial weight, g	59	59	60	59	60	60
Weight gain, g	53±10*	99±17	152±21	106±9	148±10	162±13
Food consumption, g	262	331	369	362	389	400
Protein efficiency ratio, g gain/g protein	2.86±0.81	3.04±0.30	2.74±0.21	4.19±0.35	3.80±0.19	2.71±0.16
Liver weight, g						
Unadjusted	4.33±0.40	6.05±1.13	8.43±1.39	6.20±1.14	7.95±1.05	8.09±1.22
Adjusted†	6.37	6.50	7.23	6.65	7.14	6.77
Kidney weight, g/pair						
Unadjusted	0.84±0.10	1.17±0.16	1.59±0.10	1.15±0.09	1.39±0.12	1.69±0.17
Adjusted	1.18	1.28	1.43	1.23	1.26	1.47
Adrenal weight, mg/pair						
Unadjusted	19.8±2.6	24.7±2.6	25.5±3.2	23.1±3.4	30.7±7.4	31.6±4.9
Adjusted	24.3	24.9	20.6	22.6	26.2	25.5
Liver lipids, %	5.02±0.62	4.50±1.07	5.67±1.18	5.50±1.15	5.14±0.64	5.47±0.80

*Standard deviation.
†Adjusted for differences in body weight.

casein were not significantly influenced by the dietary protein level, whereas those found with fish flour were significantly different at each protein level tested. It is well known that proteins of high nutritional value give maximum P.E.R. values at relatively low dietary protein levels, and show decreasing values as the dietary protein level is increased above the needs for growth and maintenance. It should be noted that the P.E.R. values found for casein in the present studies were higher than those reported previously by Chapman *et al.* (7), using a different strain of rats. It has recently been shown (11) that the strain of rats used significantly influences P.E.R. values.

The data on organ weights in experiment 1 (Table I) were examined by analysis of covariance, to take into account differences in body weight. It should be noted, in this connection, that the common practice of expressing organ weights as a fraction or percentage of body weight is often not justified, since it assumes that the part is directly proportional to the whole. As Heroux and Gridgeman (12) and others have pointed out, this assumption is satisfactory if the part is large, such as muscle mass, but is questionable if the part is small, such as adrenal glands. The liver weights found for the various groups in experiment 1 did not differ significantly when adjusted for differences in body weight of the animals. The adjusted kidney weights for the various groups were found to differ significantly, however, with those for the animals fed the diets containing 15% protein (diets 3 and 6) being larger than those for the animals fed the other diets. Hypertrophy of the kidneys in response to protein has been noted previously by other investigators (13, 14). The adrenal glands in experiment 1 did not differ significantly in weight from group to group, when adjusted for differences in body weight.

Liver lipid levels found in experiment 1 (Table I) varied from 4.50% to 5.67% and were apparently not influenced by the diet fed.

Supplementation of Whole Wheat Flour with Fish Flour

The results of experiment 2, in which small amounts of fish flour were used to supplement whole wheat flour, are presented in Table II. Addition of 2, 4,

TABLE II

Effect of supplementation with defatted fish flour on the nutritional value of whole wheat flour, as measured in growing male rats fed diets containing 10% protein for 4 weeks (experiment 2)

	Diet 1	Diet 2	Diet 3	Diet 4
Fish flour added, lb/100 lb wheat flour	0	2	4	6
Initial weight, g	54	54	54	54
Weight gain, g	37±7*	67±10	84±14	102±10
Food consumption, g	249	314	337	365
Protein efficiency ratio, g gain/g protein	1.49±0.12	2.12±0.13	2.48±0.15	2.80±0.11
Liver weight, g				
Unadjusted	3.68±0.49	4.99±0.83	5.42±0.93	6.41±0.80
Adjusted†	4.98	5.24	5.02	5.33
Kidney weight, g/pair				
Unadjusted	0.88±0.17	1.01±0.06	1.07±0.16	1.17±0.13
Adjusted	1.10	1.05	1.00	0.99
Adrenal weight, mg/pair				
Unadjusted	20.2±3.4	21.6±3.4	22.7±3.6	23.6±3.6
Adjusted	22.8	22.1	21.9	21.4
Liver lipids, %	7.56±1.50	7.23±1.17	7.97±2.82	6.99±0.64

*Standard deviation.
†Adjusted for differences in body weight.

or 6 lb of fish flour per 100 lb of whole wheat flour increased the P.E.R. in a stepwise fashion, from 1.49 to 2.80. Sure (4) found that small amounts of fish flour increased the P.E.R. value found with whole wheat, although, in his studies, the dietary protein level was not kept constant. The weights of the livers, kidneys, and adrenal glands of the animals fed the various diets did not differ significantly when adjusted by covariance analysis for differences in body weight. Liver lipid levels did not differ significantly from group to group, but were higher than those found with casein or fish flour in experiment 1.

Addition of Fish Flour to Bread

The results of experiment 3 are given in Table III. The two bread samples which contained fish flour with or without milk had protein (N×6.25) contents

TABLE III

Effect of defatted fish flour (DFF) on the nutritional value of white bread, as measured in growing male rats fed diets containing 10% protein for 4 weeks (experiment 3)

	Diet 1, casein	Diet 2, bread	Diet 3, bread + 10% DFF	Diet 4, milk bread	Diet 5, milk bread + 10% DFF
Initial weight, g	40	40	39	40	40
Weight gain, g	109±14*	23±8	145±16	43±10	130±13
Food consumption, g	311	177	376	216	363
Protein efficiency ratio, g gain/g protein	3.49±0.14	1.30±0.21	3.87±0.13	1.97±0.27	3.59±0.18
Liver weight, g					
Unadjusted	6.47±0.55	2.74±0.44	7.71±0.75	4.27±0.57	7.24±0.57
Adjusted†	6.00	4.38	6.36	5.42	6.26
Kidney weight, g/pair					
Unadjusted	1.18±0.08	0.85±0.07	1.53±0.16	0.92±0.09	1.35±0.10
Adjusted	1.15	0.96	1.44	1.00	1.29
Adrenal weight, mg/pair					
Unadjusted	22.5±4.2	13.9±2.2	23.8±3.3	18.7±2.3	25.2±2.8
Adjusted	22.4	14.2	23.5	18.9	25.0
Liver lipids, %	5.48±0.30	8.38±0.87	6.17±1.04	9.20±2.10	6.30±1.15
Liver cholesterol, mg/g wet tissue	2.50±1.38	3.71±0.57	2.86±0.89	2.94±1.18	2.13±1.31

*Standard deviation.
†Adjusted for differences in body weight.

of 18.0 and 16.5%, respectively (air-dry basis). These breads showed reduced loaf volume, were slightly darker in color, and had a somewhat "rubbery"

texture, as compared to the normal breads, but the smell and taste were not noticeably affected. The P.E.R. of white bread was significantly increased by addition of 4.2% milk solids (diet 2 vs. diet 4). Addition of fish flour to bread made with or without milk increased the P.E.R. values by 82% and 198%, respectively.

When adjusted for differences in body weight by analysis of covariance, the adrenals, kidneys, and livers of the animals in the various groups were not significantly different in weight. Liver lipid levels, however, were significantly higher in the animals which received diets containing bread with or without milk (diets 2 and 4) than in those fed diets containing bread with added fish flour (diets 3 and 5). Liver cholesterol levels also tended to be higher in the animals fed diets 2 and 4 than in those fed the remaining diets, although the differences were not statistically significant.

The higher liver lipid levels found in the animals fed bread diets without added fish is probably related, in part, to the fact that these diets contained a relatively low level (10%) of protein of rather poor nutritional value. It is well known, from the work of Litwack *et al.* (15), Harper *et al.* (16), and others, that deficiencies in the quality and/or quantity of dietary protein may result in deposition of excess amounts of liver fat.

Lysine values found for the flour and bread diets in experiments 2 and 3 are summarized in Table IV. Addition of increments of fish flour to whole wheat

TABLE IV

Effect of defatted fish flour on lysine content of bread and flour proteins

Diet	Lysine content, g/16.0 g N
Experiment 2	
Whole wheat flour	3.02
+ 2 parts fish flour	3.63
+ 4 parts fish flour	4.37
+ 6 parts fish flour	4.68
Experiment 3	
White bread	2.43
+ 10% fish flour	6.32
Milk bread (4.2% milk solids)	3.16
+ 10% fish flour	5.86

flour in experiment 2 increased the lysine content from 3.02 g per 16.0 g of nitrogen to 4.68 g per 16.0 g N, in a stepwise manner. The white bread used in experiment 3 (diet 2) contained 2.43 g of lysine per 16.0 g N. Addition of 4.2% milk solids (diet 4) increased the lysine content to 3.16 g per 16.0 g N. The values found for the bread samples which contained fish flour, with or without milk solids, were 5.86 g and 6.32 g lysine per 16.0 g N, respectively.

The percentage of lysine in the protein of the flour and bread diets in experiments 2 and 3 was plotted against P.E.R. in Fig. 1. A highly significant correlation coefficient of 0.993 was found, indicating that the P.E.R. values for these diets are a direct function of the percentage lysine in the protein. Rogers *et al.* (17) and Flodin (18) have also observed a significant relationship between

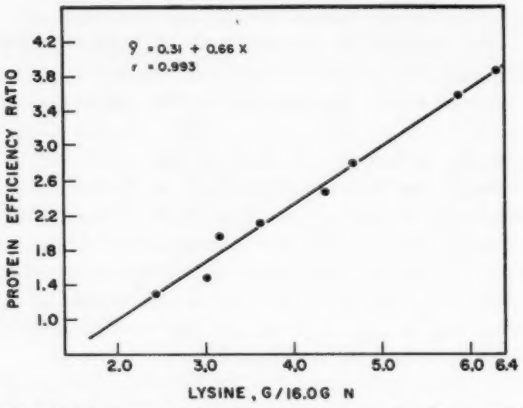

FIG. 1. Relationship between lysine content of bread and flour proteins and P.E.R. values.

lysine concentration and P.E.R. for foods low in lysine. Howard *et al.* (19) have recently suggested that an ideally balanced "complete" protein should contain 5.3 g of lysine per 16.0 g N. For the present data, this would correspond to a P.E.R. value of 3.19, similar to that for casein.

The supplemental value of fish flour, based on its content of "complete" protein, was calculated according to the formula given by Howard *et al.* (19) and found to be 185. Further calculations indicated that addition of 2 lb of fish flour to 100 lb of whole wheat flour (as in experiment 2) increased the crude protein content of the mixture by approximately 10%, but increased the "complete" protein content by approximately 42%. Since fish flour contains approximately 10.9 g lysine per 16.0 g N (17), its supplemental value for proteins deficient in lysine is very high, because, in addition to the "complete" protein which it supplies, it also contributes a considerable amount of "extra" lysine.

It is of interest to calculate the protein rating of bread containing fish flour, by the procedure given by Campbell and Chapman (20), which involves multiplication of the corrected P.E.R. of the food by the grams of protein supplied in a "reasonable daily intake" of the food. The rating for white bread (diet 2), was found to be 11.1, whereas that for similar bread containing 10% fish flour (diet 3) was 51.5.

It may be concluded that fish flour is an excellent source of high quality protein, and should be of particular value in supplementing diets deficient in lysine.

Acknowledgments

The authors are indebted to Dr. H. Fougere, of the Fisheries Research Board of Canada, for the sample of fish flour tested; to Miss Ruth G. MacIntosh, of the Department of Fisheries, Government of Canada, for baking the bread samples; and to Mr. C. Perusse and Mr. M. Airth for technical assistance.

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CHANGES IN THROMBIN GENERATION AND ANTITHROMBIN TITER FOLLOWING MASSIVE BLEEDING AND TRANSFUSION IN DOGS[1]

F. C. MONKHOUSE AND SUSAN MILOJEVIC

Abstract

Thrombin generation, plasma antithrombin levels, and plasma protein levels were measured in dogs following rapid massive bleeding and transfusion. Three groups of four dogs per group were used. One group was transfused with saline, one with dextran, and one with a plasma fraction. The plasma fraction was prepared from mixed dog plasma by a procedure designed to remove most of the known clotting factors with a minimum of damage to other proteins. The in vivo dilution of blood by transfusion with two volumes of saline or one of plasma fraction increased the *rate* of thrombin generation. Dilution by transfusion with dextran decreased both rate and amount of thrombin generation. For the first few hours following transfusion with saline, plasma antithrombin increased more rapidly than total plasma protein. This was probably a result of increased lymph flow. Fluctuations in the coagulability of the blood were observed up to 72 hours following bleeding and transfusion. The fluctuations following saline transfusion were less prolonged than when dextran or plasma fraction was used.

Under normal conditions the blood remains fluid within the blood vessels despite the continuous turnover of plasma proteins involved in coagulation. Evidently a delicate balance exists between the rate at which coagulants are activated and neutralized. It is only when this balance is disturbed, locally or generally, that the signs of intravascular clotting occur.

It has been known for some time (1) that simple dilution of plasma in vitro is sufficient to shift the equilibrium towards increased coagulability. Recent experiments (2) have shown that this is due, at least in part, to an increased thrombin generation. Consideration of these results led us to investigate changes in coagulability when the equilibrium in vivo is upset by hemorrhage and transfusion. Changes in thrombin generation and antithrombin levels have been measured following transfusion with saline, dextran, and a plasma fraction. The plasma fraction was prepared from mixed homologous plasma in a manner designed to remove most clotting factors with a minimum of change in the remaining proteins. It was not possible to remove all the antithrombin activity.

Methods and Materials

Antithrombin assay.—This was carried out according to the method of Monkhouse *et al.* (3). A highly purified thrombin produced by citrate activation of prothrombin kindly supplied by the Ortho Research Foundation through the courtesy of Dr. H. O. Singher was used.

Thrombin generation.—This was carried out following the principle outlined by Pitney and Dacie (4). To 0.8 ml of plasma, 0.1 ml of saline and 0.1 ml of $CaCl_2$ (0.2 *M*) were added. At 1-minute intervals thereafter, 0.1-ml aliquots of this incubation mixture were added to 0.2 ml of fibrinogen and the clotting

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Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario.

time measured. The thrombin concentration of these aliquots was expressed in arbitrary units in reference to a standard graph.

Total protein.—This was estimated following nitrogen determinations carried out by the micro-Kjeldahl technique.

Bleeding and transfusion.—Mongrel dogs of from 8 to 10 kg were anesthetized with Nembutal. The jugular vein and carotid artery of one side were cannulated. The jugular cannula was connected with a transfusion bottle. From the arterial cannula the blood was allowed to flow into sterilized 250-ml centrifuge tubes. An amount of blood equal to 4.5 – 5% of the body weight was rapidly removed. Immediately after this transfusion, fluid was allowed to enter via the jugular vein cannula at a relatively rapid rate (30–45 ml/minute) until the required volume had been given. In the animals given saline the volume was *twice* that of the blood removed. Those receiving dextran and plasma fraction received a volume *equal to* the volume of blood removed. Approximately one hour later the bleeding and transfusion procedures were repeated. This time the unwashed cells obtained from the first bleeding were added to the transfusion fluid. The cells were obtained by centrifuging the blood for 15 minutes at 2000 r.p.m. Approximately the same volume of blood was removed and fluid transfused as in the first bleeding.

Fibrinogen.—This was prepared from bovine plasma by the method of Monkhouse and Clarke (5).

Dextran.—This was supplied through the courtesy of Dr. A. F. Charles of the Connaught Laboratories. The molecular weight of this dextran was circa 120,000.

Plasma fraction.—From 4 to 6 liters of mixed dog plasma were processed at one time. Fibrinogen was removed by heating to 54° C, maintaining that temperature for 4 minutes, quickly cooling, and centrifuging. The supernate was absorbed with barium carbonate to remove prothrombin and with aluminum hydroxide to remove most of the antithrombin. The plasma was then heated at 60° C for 2 hours. Following this treatment it was dialyzed for 2 hours against frequent changes of physiological saline. After dialysis it was centrifuged and stored at 20° C. Just prior to transfusion it was thawed and to each liter was added 400,000 units of penicillin. The protein concentration at this stage averaged 2.18%.

Results

Thrombin generation studies on the experimental animals revealed that three sets of measurements were of value in determining changes in coagulability: (*a*) the concentration of thrombin at the peak of generation, which is a measure of the *potential thrombin activity* available, (*b*) the length of time thrombin activity continues in the incubation mixture, (*c*) the length of time required for thrombin concentration to reach a peak. Changes in the last two measurements seem to be closely related, though not always identical. They reflect the rate of *thrombo*-plastin formation and/or its activity (2).

Immediate Changes (0 – 6 hours) in Thrombin Generation

The concentration of thrombin at the peak of generation is shown in Fig. 1. Zero time refers to immediately before bleeding. The second groups of samples

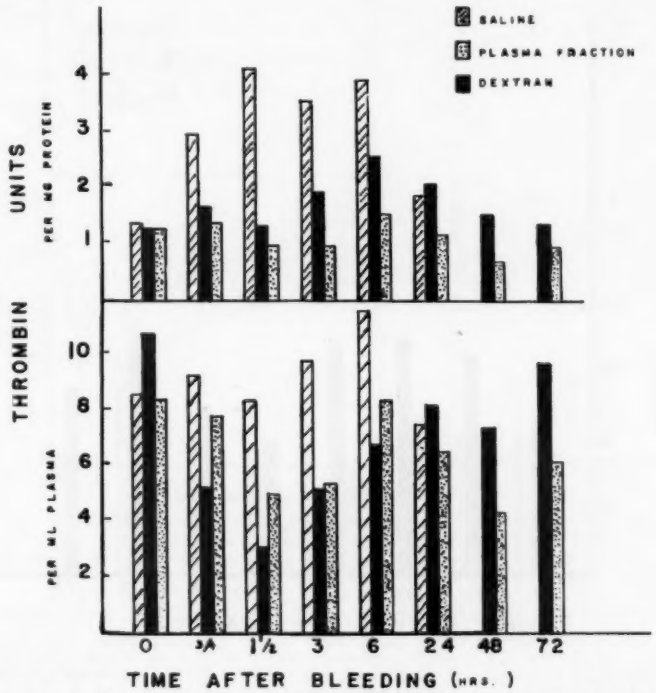

FIG. 1. Units of thrombin at the point of peak generation in samples of blood taken at various times after bleeding and transfusion. Each bar represents the mean value from four dogs. Zero time is immediately before the first bleeding.

were taken following the first transfusion and the third group of samples immediately following the second transfusion. With saline the maximum amount of thrombin per milliliter of plasma remained relatively constant, while in terms of milligrams of protein it was significantly increased. Thus the effect of transfusion with saline was similar to the effect when dilution was carried out in vitro (2). For the first 2 hours after transfusion with dextran, there was a drop in the maximum amount of thrombin generated per milliliter of plasma but relatively no change per milligram of protein. In the 6-hour samples the amount of thrombin generated per milligram protein is twice that of the normal sample. Expressed in terms of milliliters of plasma the amount of thrombin generated is much below the normal sample, though double that of the sample taken at 1½ hours. Transfusion with the plasma fraction resulted in a decrease in peak thrombin activity per milliliter of plasma and per milligram of protein. Normal values were obtained at 6 hours.

The changes in the *rate* of thrombin generation are shown in Fig. 2. It can be seen that transfusion with dextran caused quite different effects from transfusion with the other solutions. Dextran *decreased* while saline and plasma fraction *increased* the *rate* of thrombin generation.

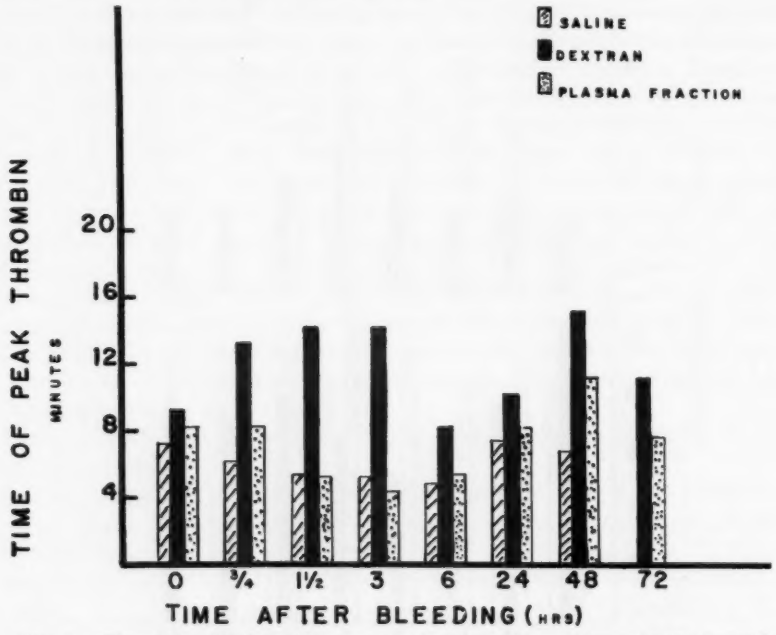

FIG. 2. Time taken for thrombin concentration to reach a maximum in the thrombin generation assay. Samples of blood were taken at various times after bleeding and transfusion. Each bar represents the mean value of four dogs.

Delayed Changes (24 – 72 hours) in Thrombin Generation

Blood samples taken at 24 hours, in all instances, showed lower than normal *peak* thrombin generation in terms of milliliters of plasma. However, the *rate* of thrombin generation was within the normal limits (Figs. 1 and 2). At 48 hours there was a significant decrease in the *rate* of thrombin generation in the samples from animals transfused with dextran and plasma fraction. There was also a sharp drop in the *peak* thrombin generation in the samples from animals receiving the plasma fraction. Both *rate* of thrombin generation and *amount* of thrombin generated were approaching normal values by 72 hours in all instances.

Antithrombin Changes

In Fig. 3 antithrombin and total protein levels are shown. Antithrombin levels immediately following the completion of transfusion decreased in proportion to the dilution of plasma proteins. The higher values following transfusion with the plasma fraction are due to the 15 to 20% antithrombin remaining in this fraction.

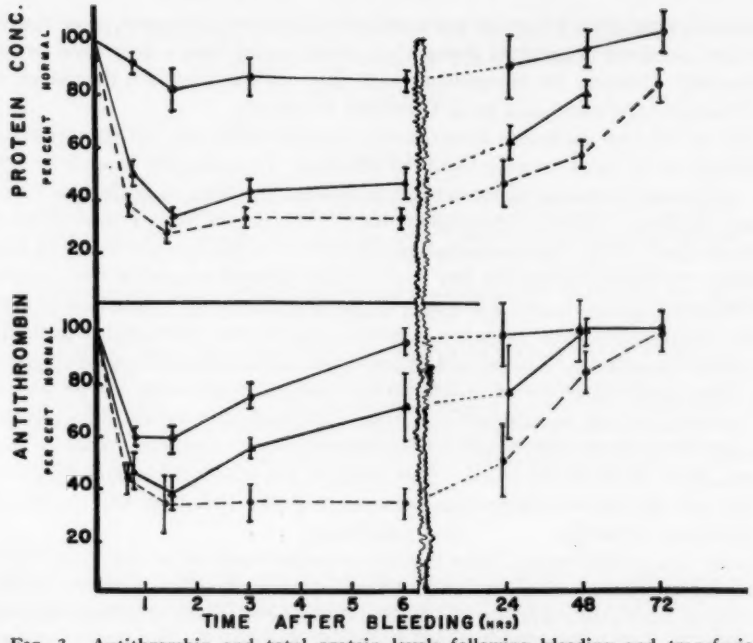

FIG. 3. Antithrombin and total protein levels following bleeding and transfusion. Mean values and standard deviations are given. Each line represents results from four animals. ●——● protein fraction; ▲——▲ saline; ○ - - - - ○ dextran.

During the first few hours after transfusion, it can be observed that, with the *saline*, the antithrombin levels increased more rapidly than the protein levels, with dextran neither antithrombin nor protein levels increased. After this initial period (4 – 6 hours) antithrombin levels increased at approximately the same rate as protein in all instances. Plasma proteins are about 80% of normal 72 hours after bleeding with both saline and dextran.

Conclusions

In a recent review, Rosenthal (6) recommended that wound or burn shock be treated with oral or intravenous administration of large volumes of physiological saline. Since dilution of plasma with saline in vitro increases the coagulability of blood (2), it seemed desirable to examine the effects of dilution in vivo. The experiments reported here show that in vivo dilution of blood by transfusion with two volumes of saline or one of plasma fraction increased the *rate* of thrombin generation as measured in the test tube. This we interpret as indicative of increased thromboplastic activity in particular and an increased tendency towards intravascular coagulation in general. It may be a result of diluting out the antithromboplastin (1). *Immediately* following the saline transfusion there was a large increase in the units of thrombin generated *per milligram of protein*, while the antithrombin activity per milligram of protein

remained constant. Thus the increased thrombin activity must have resulted from an increased amount of thrombin formed rather than a decreased amount destroyed. Dilution by transfusion with dextran resulted in a decreased *rate* of thrombin formation and total thrombin formed.

During the first few hours after transfusion with saline the antithrombin level increased much more rapidly than the protein. This increase in antithrombin per milligram of protein did not occur following transfusion with dextran and plasma fraction. Many years ago Howell (7) reported that lymph from the thoracic duct of the dog contained antithrombin in the same amount as blood plasma. An increased lymph flow is the simplest explanation for the increased antithrombin activity per milligram of protein following saline and suggests that antithrombin may be fairly generally distributed throughout the extracellular compartment. This would be consistent with findings in our own laboratory that large amounts of antithrombin concentrates are required to raise the general level of antithrombin. Also, a considerable decrease in the total amount of antithrombin would be required before a significant change in its plasma level could be detected. This may be the reason why it is difficult to to find any consistent relationship between antithrombin titer and thrombotic tendencies or episodes.

Under the conditions of these experiments fluctuations in the coagulability of the blood were observed up to 72 hours following bleeding and transfusion. The effects with saline were less prolonged than with dextran or plasma fraction. All animals were transfused immediately following the loss of blood and therefore probably should not be considered in shock. Nevertheless it was observed that the animals which received two volumes of saline were much more active at 24 hours than were animals from the other two groups. Indeed three of the four dextran-treated animals were unable to stand 24 hours after the transfusion.

Acknowledgments

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THE OXIDATION OF SUCCINATE IN EXTRACTS OF XANTHOMONAS PHASEOLI[1]

N. B. MADSEN

Abstract

Succinoxidase and succinic dehydrogenase were found in cell-free extracts of *Xanthomonas phaseoli*, obtained by sonic oscillation, and remained largely in the supernatant solution after ultracentrifugation. The effect of time of exposure of the cells to sonic oscillation on cell breakage was found to follow first order reaction kinetics, as was the "solubilization" of succinic dehydrogenase and succinoxidase. It appears that the two enzymatic activities are released from the cell in a particulate form which is further fragmented on continued treatment in the sonic oscillator.

The clear supernatant solution obtained after ultracentrifugation of the cell-free extract was found to contain those members of the electron transport chain which had previously been found in the intact cell, namely, flavoprotein and cytochromes b_1, a_1, and a_2. These substances could be reduced by the addition of succinate. Malonate prevented this reduction. The effects of various inhibitors on the succinoxidase system and on succinic dehydrogenase are presented and discussed in relation to the operation of the electron transport chain in the oxidation of succinate by this organism.

Introduction

Previous work from this laboratory has been concerned with the intermediary metabolism of the plant pathogenic organism *Xanthomonas phaseoli*. In the course of this work it has been established that cell-free extracts of the organism contain the individual enzymes of the tricarboxylic acid cycle and that the latter is probably the main pathway for the terminal oxidation of carbohydrate (1). When the organism is grown on acetate, the glyoxylate cycle is also present. Since succinate is a key intermediate in both of these metabolic cycles, a detailed study of the oxidation of this substance was undertaken in order to add to the available knowledge concerning energy transformations and electron transport systems of this plant pathogen. Hochster and Nozzolillo (2) have reported previously on a study of the respiratory carriers and DPNH oxidase in *X. phaseoli*.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: phenazine methosulphate (Sigma Chemical Co.); antimycin A (Wisconsin Alumni Research Foundation); Amytal (Eli Lilly and Co.). The following substances were gifts to Dr. R. M. Hochster and the donors are listed in parentheses: Heptyl-N-oxide* (Dr. J. W. Lightbown, National Institute for Medical Research, London); Chlorpromazine (Dr. Guy Marier, Poulenc Ltd.,

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Contribution No. 494, from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Central Experimental Farm, Ottawa, Ontario.

*The following abbreviations are used: Heptyl-N-oxide, 2-heptyl-4-hydroxyquinoline-N-oxide; DPNH, reduced diphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Tris, tris (hydroxymethyl)aminomethane.

Montreal); quinacrine (Atabrine) (Dr. M. L. Tainter, Sterling-Winthrop Research Institute, Rensselaer, N.Y.).

Enzyme Preparations

Xanthomonas phaseoli (XP8) was grown in a medium consisting of 1% yeast extract and 2% glucose. A 50-ml portion of this medium (in a 300-ml Erlenmeyer flask) was inoculated from a yeast extract – glucose – $CaCO_3$ – agar slant culture and incubated for 18 to 24 hours at 26° C on a rotary shaker. The inoculum thus obtained was then added to 700 ml of the medium contained in a 2800-ml Fernbach flask and again incubated in the above manner. Cells were harvested by centrifugation at 4000 r.p.m., washed twice with 0.9% NaCl, and suspended in two volumes of 0.1 *M* Tris buffer (pH 7.7) containing 0.01 *M* glutathione. Each batch of cells was tested for strain purity by the specific phage test (3).

The "whole extract" was prepared by treatment of the cell suspension (usually 20 to 25 ml) in a Raytheon 200-w sonic oscillator for various times (usually 5 minutes) at a power output of 0.9 amp and a temperature of 0 to 5° C, followed by centrifugation at 15,000 × *g* for 10 minutes at 5° C to remove unbroken cells and cellular debris. The major portion of the work in this paper was done with the supernatant solution obtained after a further centrifugation of the whole extract for 90 minutes at 40,000 r.p.m. (103,000 × *g*) in a Spinco model L preparative ultracentrifuge and this preparation will be referred to as "extract".

Enzyme Assays

Succinoxidase activity was determined manometrically. Each Warburg vessel contained 100 μmoles potassium phosphate buffer, pH 7.5 to 7.7, and a suitable amount of extract (usually 0.2 ml) containing 4 mg protein in a volume of 2.3 ml. The side arm contained 60 μmoles succinic acid, which had been neutralized with KOH, in 0.5 ml. Potassium hydroxide (0.2 ml of a 4 *N* solution) and filter paper were routinely placed in the center well.

Succinic dehydrogenase was measured by the manometric method of Singer and Kearney (4). The main compartment of the Warburg vessel contained 100 μmoles potassium phosphate buffer, pH 7.5 to 7.7, 25 μmoles NaCN, and 0.2 ml extract in a volume of 2.5 ml. The side arm contained 60 μmoles succinate and 2 mg phenazine methosulphate in 0.5 ml. All incubations were at 30° C.

Experimental and Results

The "Solubilization" of Succinoxidase and Succinic Dehydrogenase

Four lots of cells weighing 8 g each were suspended in twice their volume of buffer, as described in "Materials and Methods", and subjected to various times of oscillation in the sonic oscillator at a power output of 0.9 amp. The resultant mixtures were centrifuged for 10 minutes at 15,000 × *g* and the well-packed precipitates weighed as a measure of the cells remaining unbroken. Whole extract and extract were then obtained as described in "Materials and Methods". The enzymatic activity of the whole extract is taken as representing

the total activity released from the broken cells (particulate plus soluble fractions), while the enzymatic activity of the extract represents the soluble activity only. The difference between the whole extract and the extract was accepted as particulate activity. A preliminary experiment in which particles obtained from the centrifugation of the whole extract at 103,000 × *g* were resuspended in the original volume showed that the method was valid for succinoxidase. In this experiment 0.2 ml of the whole extract took up 62 μl. of oxygen in 10 minutes, the extract took up 38 μl. and the particles 23 μl. (In this experiment the amount of protein contained in each 0.2-ml aliquot was 6.4 mg for the whole extract, 3.9 mg for the extract, and 2.1 mg for the particles.)

Figure 1 shows the course of release of succinoxidase activity from the cells during sonic oscillation. The activity appeared first in the particulate fraction and was then found chiefly in the soluble phase with continuing treatment. That succinoxidase was originally particulate was confirmed by finding that when extracts prepared by grinding with alumina (Alcoa A-301) were ultracentrifuged, virtually none of the succinoxidase activity was recovered in the supernatant solution. A similar result was obtained by measurement of the succinic dehydrogenase activity.

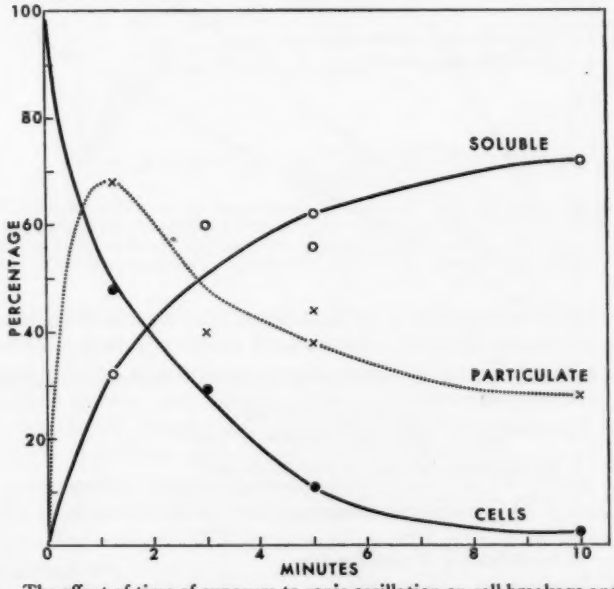

FIG. 1. The effect of time of exposure to sonic oscillation on cell breakage and distribution of succinoxidase.
●——● Percentage of cells remaining unbroken.
○——○ Succinoxidase activity in supernatant solution after ultracentrifugation as percentage of total activity in the whole extract.
×——× Succinoxidase remaining particulate (by difference). The two points at 5 min are from different experiments. A preliminary experiment, conducted at 5 min only, provided the upper point on the "soluble" curve and the lower point on the "particulate" curve.

Figure 2 is a first-order reaction plot of the effect of time of exposure to sonic oscillation on the extent of cell breakage, on the "solubilization" of succinic dehydrogenase, and on that of succinoxidase. It may be seen that all three effects follow first-order reaction kinetics. Hochster and Nozzolillo (2) examined the DPNH oxidase activity of these same fractions and have reported a similar first-order relationship. Although the succinoxidase activity remains soluble in the supernatant solution after ultracentrifugation, it could not be fractionated readily with ammonium sulphate. All of the activity precipitated at 30% saturation of ammonium sulphate.

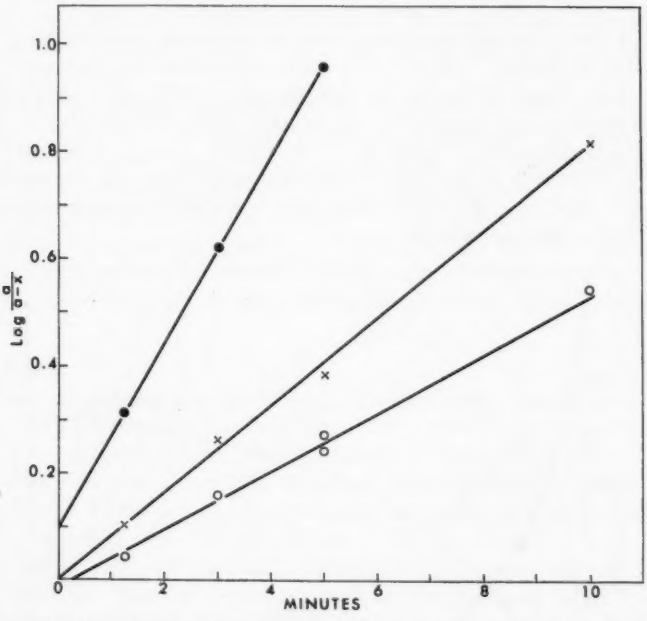

FIG. 2. First-order plot of the effect of time of exposure to sonic oscillation on:
(*a*) ●——● Breakage of cells.

$$\text{Log}\frac{a}{a-x} = \log\frac{\text{original weight of cells}}{\text{weight remaining unbroken at time } t}.$$

(*b*) ×——× Solubilization of succinic dehydrogenase.

$$\text{Log}\frac{a}{a-x} = \log\frac{\text{activity of whole extract at 10 min}}{\text{activity of whole extract at 10 min minus activity in supernatant at time } t}.$$

(*c*) ○——○ Solubilization of succinoxidase.

$\text{Log}\frac{a}{a-x}$ is defined as for (*b*) above.

Kinetic Studies

The succinoxidase was found to have a broad pH optimum between pH 7.5 and 8.0. Since the oxidase was found to be soluble by the criterion of remaining in the supernatant layer after ultracentrifugation, it was felt that the primary

dehydrogenase, presumably a flavoprotein, might pass electrons directly to oxygen, forming hydrogen peroxide. The stoichiometry of the oxygen uptake was therefore investigated. Figure 3 shows that one-half mole of oxygen is

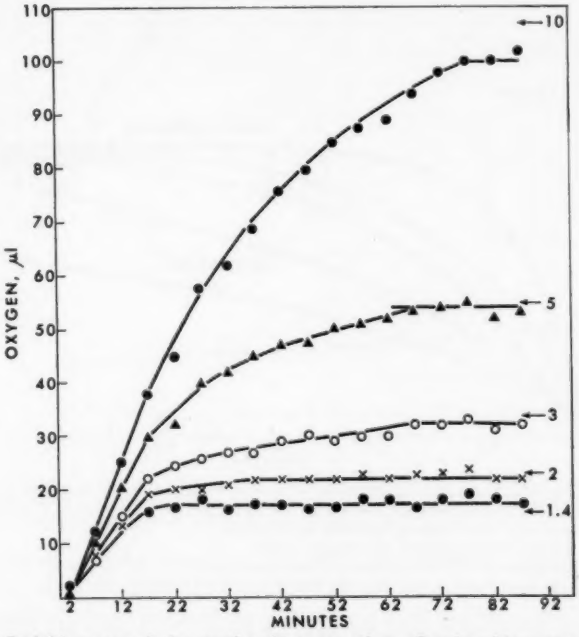

FIG. 3. Stoichiometry of the succinoxidase reaction. Succinoxidase was determined by the usual procedure (using 0.2 ml of extract) with varying amounts of succinate. The figures on the right indicate the number of µmoles of succinate added and the arrows indicate the theoretical oxygen uptake for 0.5 mole of O_2 per mole of succinate.

taken up per mole of succinate present. The extracts contain a very powerful catalase which might decompose the hydrogen peroxide formed to yield such a stoichiometry. The stoichiometry was therefore tested under conditions in which catalase activity was strongly inhibited and the results are plotted in Fig. 4. It may be seen that catalase activity could be effectively inhibited by concentrations of sodium azide which did not change the stoichiometry. In addition, no peroxide could be detected by the method of Savage (5), nor did the addition of ethanol to the usual reaction mixture result in increased oxygen uptake (6). The enzyme system present in the extract is, therefore, most likely a true oxidase.

Lineweaver–Burk plots for the activity of the oxidase with succinate and of the dehydrogenase with succinate and phenazine methosulphate are shown in Fig. 5. The Michaelis constants (K_m's) of the two activities for succinate are essentially the same, 1.16×10^{-3} M for the oxidase and 0.9×10^{-3} M for the dehydrogenase. These values agree well with those found by Singer and his colleagues for preparations of succinic dehydrogenase from both animal and

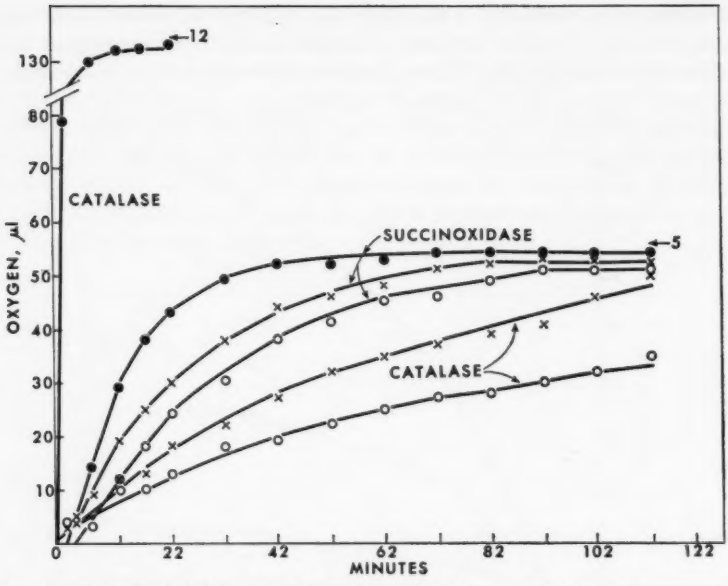

FIG. 4. The effect of azide on catalase or succinoxidase. Activities were measured in the presence of 12 μmoles H_2O_2 or 5 μmoles succinate and the theoretical oxygen release or uptake (respectively) for these quantities is indicated.
●——● Uninhibited catalase or succinoxidase.
×——× Catalase or succinoxidase in the presence of 0.01 *M* NaN_3.
○——○ Catalase or succinoxidase in the presence of 0.02 *M* NaN_3.

bacterial sources (7, 8) and with that of Chance, obtained with a modified Keilin–Hartree preparation of succinoxidase (9). The K_m of the dehydrogenase for phenazine methosulphate (2×10^{-4} *M*) is, however, less than the 5×10^{-4} *M* reported by Singer (7) for the beef heart enzyme.

The effects of various inhibitors on the oxidase and on the dehydrogenase are compared in Table I. Certain inhibitors which might be expected to act on the primary dehydrogenase (i.e., the enzyme which has succinate as its substrate) are seen to inhibit both the dehydrogenase and the oxidase activities.

TABLE I

Inhibitors of succinoxidase and succinic dehydrogenase

Inhibitor	Molar concentration	Percent inhibition of: Succinoxidase	Succinic dehydrogenase
CN—	5×10^{-3}	87	0
NaN_3	2×10^{-2}	50	12
Amytal	6.6×10^{-3}	65	8
o-Phenanthroline*	2×10^{-3}	50	43
NH_2OH	1×10^{-2}	70	0
Chlorpromazine	5×10^{-3}	100	90
Antimycin A	1×10^{-4}	8	7
Heptyl-N-oxide	8×10^{-6}	0	0
Malonate	1×10^{-2}	85	92

*This inhibition was determined on a preparation which had been precipitated with ammonium sulphate.

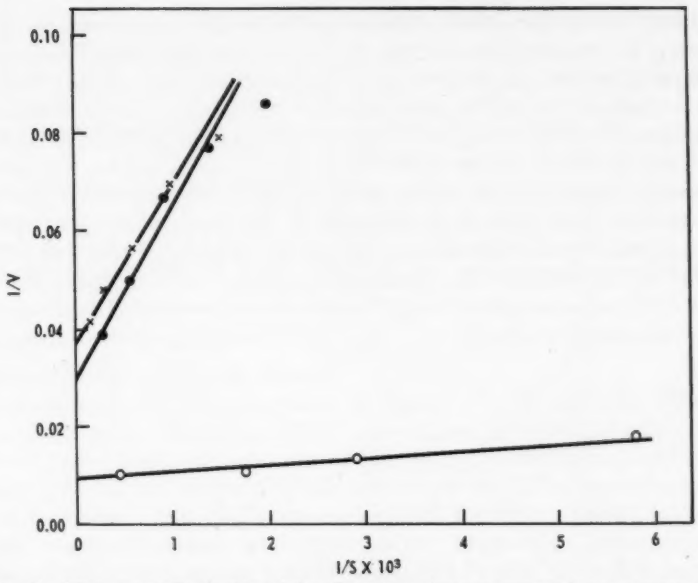

FIG. 5. Lineweaver–Burk plots of the effect of succinate concentration on succinoxidase activity (●——●) or succinic dehydrogenase activity (×——×); and of phenazine methosulphate concentration on succinic dehydrogenase activity (○——○).

Of these, malonate is the classical competitive inhibitor of succinate oxidation (10). Singer showed that *o*-phenanthroline produced a partial inhibition of purified succinic dehydrogenase by combining with the iron contained in the enzyme (11). This compound did not inhibit the activities in the extract of *X. phaseoli* but after precipitation with ammonium sulphate it was possible to obtain the inhibition shown in Table I. Both the oxidase and the dehydrogenase are inhibited about equally and this may indicate that iron is also a component of the bacterial enzyme.

Table I shows that those inhibitors which are known to act on the electron transport chain, such as cyanide, azide, and Amytal, have little or no effect on the dehydrogenase but cause considerable inhibition of the oxidase. Cyanide, azide, and hydroxylamine are usually considered to affect cytochrome oxidase (12) and in the present case may be acting on the cytochrome a_1 and (or) a_2 found in this organism (2). Cyanide and Amytal were also found to inhibit the DPNH oxidase of *X. phaseoli*, but azide and hydroxylamine did not (2). Antimycin A and heptyl-N-oxide are believed to act between cytochromes *b* and *c* (13, 14) and these substances did not cause any inhibition here. Antimycin A was previously found not to inhibit cell-free extracts of *Escherichia coli* and *Staphylococcus aureus* oxidizing succinate or cytochrome (15), or cell-free extracts of *X. phaseoli* oxidizing DPNH (2). Heptyl-N-oxide was also found not to inhibit the DPNH oxidase of *X. phaseoli* (2). Lightbown and Jackson showed that this compound was effective in inhibiting the succinoxidase of *E. coli* (15) and so its failure to inhibit the oxidases of our organism is puzzling

since these two species exhibit difference spectra similar enough (16, 2) that they may be presumed to contain similar cytochrome components. It is possible that bacterial species may exhibit differences between their cytochrome systems which are too subtle to be picked up with current spectrophotometric techniques. The inhibition with Chlorpromazine is similar to that reported for the DPNH oxidase of this organism (2).

It was found also that the oxidase activity could be titrated against increasing concentrations of Amytal, as is illustrated in Fig. 6. Chance (17) has shown that in animal mitochondria this compound prevents the transfer of electrons from DPNH to flavoprotein. On the other hand, Estabrook (18) found that

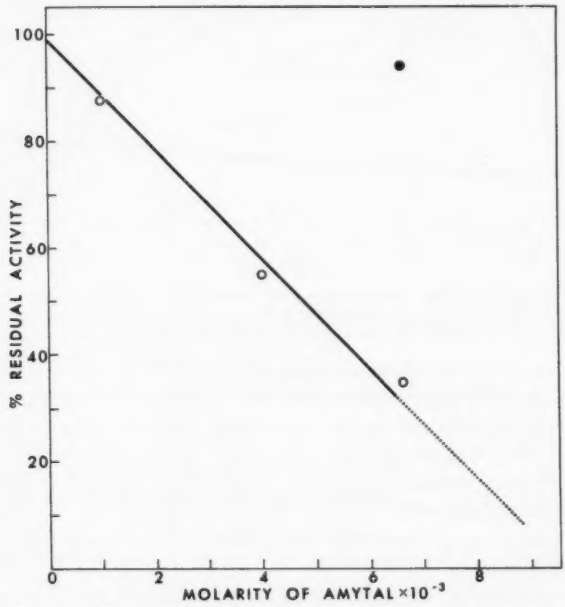

FIG. 6. The titration of succinoxidase activity with Amytal.

TABLE II

Effect of quinacrine and flavins on succinoxidase*

Molarity of quinacrine	Molarity of flavin			
	0	0.002	0.005	0.01
	Experiment 1. Flavin mononucleotide			
0	100	133	136	136
0.0033	31	50	75	95
0.0066	27	38	49	62
0.01	15	26	32	
	Experiment 2. Flavin adenine dinucleotide			
0	100	127	127	123
0.0033	33	56	79	95

*The values given represent the activity of succinoxidase as a percentage of the activity in the absence of either quinacrine or flavin.

in fragmented mitochondria flavoprotein could be reduced by DPNH but that electrons could not be passed from flavoprotein to cytochrome *b*. It is probable that the latter situation obtains in our extracts but it has not yet been possible to determine the site of action of Amytal in the *X. phaseoli* system.

Quinacrine has long been known to inhibit flavoproteins and the inhibition of D-amino acid oxidase is an example that has been thoroughly investigated (19). Haas showed that other parts of the electron transport chain can also be inhibited, especially cytochrome reductase and cytochrome oxidase (20). Table II indicates that the succinoxidase of *X. phaseoli* is activated about 30% by either FMN or FAD. Quinacrine inhibits the oxidase activity and this inhibition is relieved by either FMN or FAD. The lack of specificity of these compounds renders uncertain any interpretation based on their action.

Difference Spectra of Cell-free Extracts

When an extract of this organism is placed in both cells of a differential spectrophotometer (Beckman DK-1) and oxygen is shaken gently into the reference cell, a spectrum consisting of the reduced cytochrome peaks and the flavoprotein trough found by Hochster and Nozzolillo (2) in the whole organism can be recorded. Presumably the endogenous substrates present reduce the electron transport system as they do in the whole cell. After exhaustive dialysis of the extract against 0.05 *M* Tris (pH 7.5) at 0° C, this spectrum can no longer be obtained upon adding oxygen to the reference cell, and a fairly straight base line is obtained. Upon the addition of succinate to the sample cell, however, the spectrum shown in Fig. 7 results. This spectrum, with a trough at 656 mμ, peaks at 635, 600, 562, 531, a trough at 462, and a peak at 430 having a shoulder at 440, is similar to the spectra found in the whole organism by Hochster and Nozzolillo (2). The specificity of succinate in reducing the cytochrome system is shown by the fact that when malonate was added to the sample cell before succinate, no such spectrum was obtained. This experiment suggests that succinate has caused the specific reduction of flavoprotein and cytochromes b_1, a_1, and a_2 and is therefore presumptive evidence that these substances are concerned with the transport of electrons from succinate to oxygen. It should be noted that the addition of DPNH to the extract will also produce the same spectrum (2). The evidence that cytochrome a_2 is reduced by succinate is of interest because Tissieres (21) found that extracts of *Aerobacter aerogenes* oxidized succinate and that succinate reduced cytochromes b_1 and a_1 in the extract, but no evidence could be found for the presence of cytochrome a_2, although it was present in the whole cells.

When the extract was fractionated by adding ammonium sulphate to 40% saturation, followed by treatment of the dissolved and dialyzed precipitate with calcium phosphate gel (3 mg dry weight of gel per mg of protein) and elution with 0.5 *M* phosphate buffer, a clear yellow solution was obtained which had negligible oxidase activity but which still retained considerable dehydrogenase activity. When this fraction was reduced with succinate and its spectrum recorded, using the non-reduced fraction as the blank, the spectrum shown in Fig. 8 was obtained. The peak at 564 mμ indicates the presence of cytochrome

b_1, while the trough at 464 indicates flavoprotein, but there is an absence of detail in the region above 580 mμ, where the alpha bands of cytochromes a_1 and a_2 are found. Thus the loss of the ability to oxidize succinate is correlated with the lack of appearance of clearly reduced bands of these two cytochromes but flavoprotein and cytochrome b_1 are still present and can be reduced by succinate. This result is in agreement with flavoprotein and cytochrome b_1

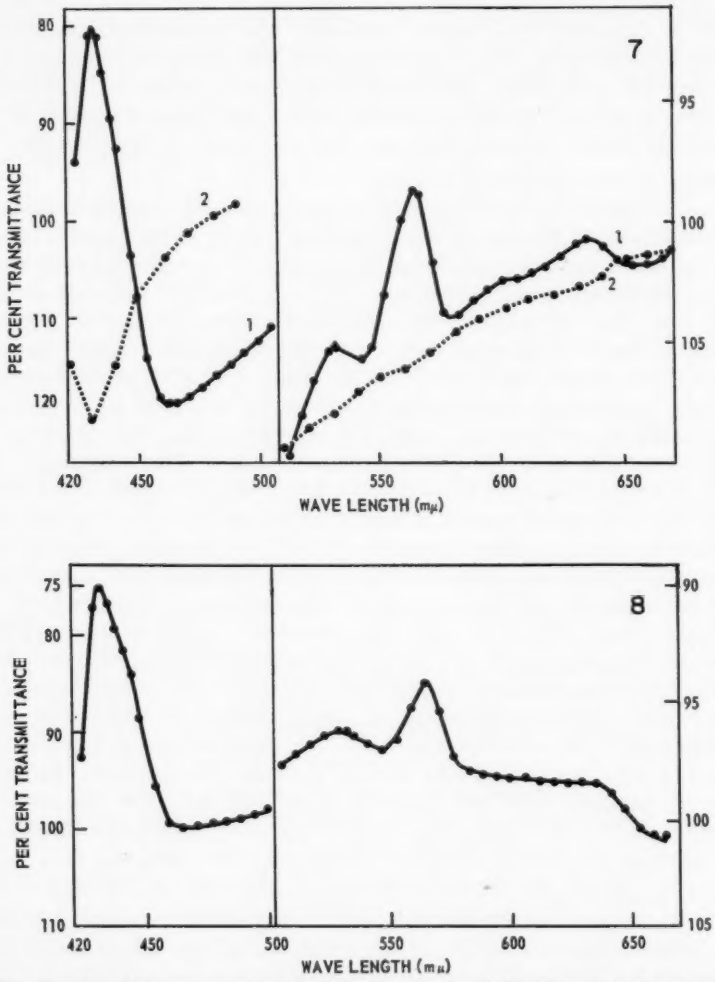

FIG. 7. The difference spectra of dialyzed extracts of *X. phaseoli* (reduced minus oxidized). Undiluted supernatant solution from ultracentrifugation was dialyzed and placed in both cells of the Beckman DK-1: (1) 150 μmoles succinate was added to the sample cell; (2) 50 μmoles malonate was added to the sample cell before adding the succinate.

FIG. 8. The difference spectrum (reduced minus oxidized) of a partially fractionated extract of *X. phaseoli*. Conditions as in Fig. 7.

being the first two steps in the electron transport chain concerned with the oxidation of succinate in this organism.

Discussion

The present investigation confirms earlier results from work with other bacterial species with regard to the finding that succinic dehydrogenase and succinoxidase are particulate (22, 23). Marr and Cota-Robles (24) studied the effect of sonic disruption on *Azotobacter vinelandii* and found that the release of various enzymes and other cell components followed exponential curves. The rate of release of glucose-6-phosphate dehydrogenase and ribonucleic acid were identical with the rate of cell rupture, suggesting that these entities were present in the cytoplasm, while the release of hydrogenase, cytochrome, and phospholipid coincided with the turbidity decrease, suggesting that the latter entities were originally present in the cell envelope. The present investigation of the effect of sonic oscillation on cell disruption and distribution of enzymic activities has provided data which are quite similar to those reported by Marr and Cota-Robles. While the present work, as well as that of other workers in the past (6, 25) suggests that sonic oscillation results in a soluble succinoxidase by the criterion that it is not sedimented in the ultracentrifuge, failure to achieve a significant degree of purification or fractionation by the conventional methods of protein purification indicates that a small particle or one with a high lipid content may be involved. Repaske (6) reported a similar difficulty in purifying a "soluble" succinoxidase from *Azotobacter vinelandii*.

The kinetics of the succinic dehydrogenase and succinoxidase studied in this organism do not appear to differ greatly from those of other species. The inhibitor studies as well as the spectra obtained by reduction of extracts with succinate suggest that the sequence of electron transfer from succinate to oxygen is very similar to that suggested by Hochster and Nozzolillo (2) for the oxidation of DPNH by the same organism. This pathway of electron transport is:

succinate→flavoprotein→cytochrome b_1→cytochrome a_1→cytochrome a_2→O_2.

Acknowledgments

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A NEW REAGENT FOR THE GUANIDINATION OF PROTEINS[1]

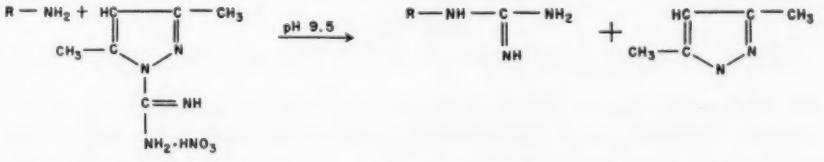
A. F. S. A. HABEEB[2]

Abstract

A reagent, 1-guanyl-3,5-dimethyl pyrazole nitrate, has been used for the guanidination of bovine serum albumin and β-lactoglobulin. The reagent is more stable and permits the guanidination to occur under more gentle conditions than the O-methyl isourea formerly employed. The modified albumin had the same sedimentation coefficient and electrophoretic mobility as the native protein. The guanidination of β-lactoglobulin required blocking of the sulphydryl groups by N-ethyl maleimide and iodoacetic acid. Guanidinated β-lactoglobulin had a lower sedimentation coefficient and greater negative electrophoretic mobility than native lactoglobulin.

Introduction

Various reagents have been used for the chemical modification of the free amino groups of proteins. Reagents that replace the positively charged NH_3^+ group by a negatively charged or neutral group may produce structural changes of some proteins (1, 2) with non-specific loss of biological activity. The reagent, O-methyl isourea (3), replaces the positively charged NH_3^+ group with a positively charged $NH—C(NH_2)_2^+$ group without changes in the net charge of the protein below about pH 8. Its main disadvantage is the high optimum pH for its reaction (about pH 10.5–11), which may be severe for some proteins. This paper describes the use of 1-guanyl-3,5-dimethyl pyrazole nitrate, which guanidinates proteins at pH 9.5. A preliminary note on this



reagent has already appeared (4). The reagent has been used by Bannard *et al.* (5) and Scott *et al.* (6) for the preparation of monoalkyl and dialkyl guanidines from aliphatic and aromatic amines.

Methods and Results

Preparation and Stability of the Reagent

The reagent, 1-guanyl-3,5-dimethyl pyrazole nitrate (GDMP), was synthesized by the method described by Bannard *et al.* (5). Since Scott and Reilly (7) found GDMP to be susceptible to hydrolytic deguanylation with the formation of 3,5-dimethyl pyrazole and urea, the stability of the reagent was examined in

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[2]National Research Council Postdoctorate Fellow 1958–1959.

borate buffer, pH 9.5, at several temperatures. The ultraviolet absorption spectrum of a solution of 0.119 μmole/ml GDMP in borate buffer, examined in a Cary recording spectrophotometer, exhibits a maximum at 234 mμ (Fig. 1);

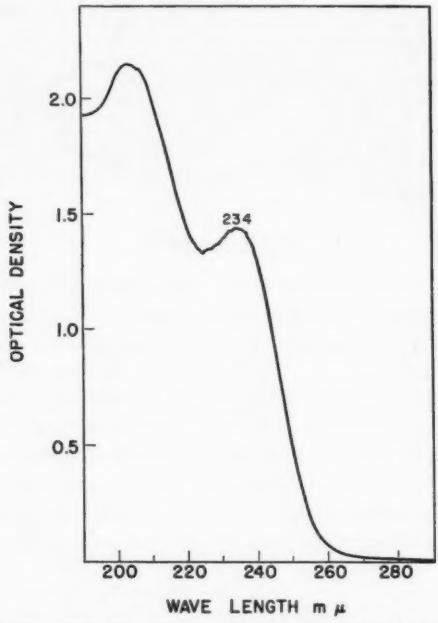

FIG. 1. Ultraviolet absorption spectrum of GDMP solution in borate buffer, pH 9.5.

the decrease at this maximum was used to follow the decomposition of the reagent. Aliquots of a fresh solution of GDMP (0.297 μmole/ml in buffer) were stored at 0°, room temperature, and 50° C. At intervals, 2-ml samples were withdrawn, diluted with buffer to 5 ml, and examined spectrophotometrically. Figure 2 illustrates that the destruction of GDMP followed a first-order reaction mechanism. While destruction was considerable at room temperature and rapid at 50° C, only about 10% was decomposed after 42 days at 0° C.

Reaction of 1-Guanyl-3,5-Dimethyl Pyrazole Nitrate with Bovine Serum Albumin

The reaction was carried out in an ice bath and studied as a function of molarity of GDMP. GDMP was dissolved in a few milliliters of water; the pH was adjusted to pH 10 by adding 1 *N* sodium hydroxide, with magnetic stirring; and portions of this solution were diluted with water to 0.1, 0.2, and 0.5 *M* GDMP. Bovine serum albumin (BSA) was dissolved in these solvents to 5% concentration and the pH readjusted if necessary. After it was stirred at 0° for 3 or 7 days, the solution was dialyzed exhaustively against phosphate buffer, pH 7.5, μ (ionic strength) 0.1. Protein concentrations were measured with a Brice-Phoenix differential refractometer. Ninhydrin colorimetric analysis (8) was used to estimate the free amino groups of the proteins with

solutions of the unmodified protein as standard. Figure 3 shows the percentage of amino groups reacted as a function of molarity of GDMP solution for 3- and for 7-day reaction periods. The reaction of 92% amino groups was obtained after 7 days in 0.5 *M* GDMP.

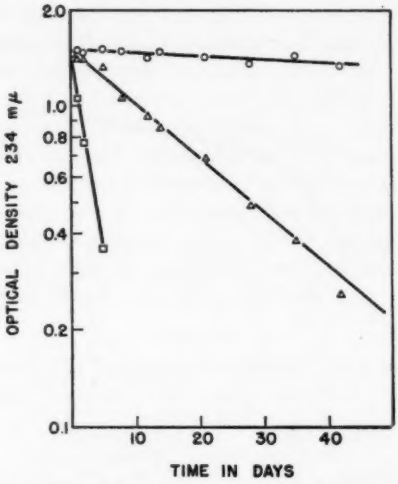

FIG. 2. Destruction of GDMP solution on storage at pH 9.5. ○ at 0° C; △ at room temperature; □ at 50° C.

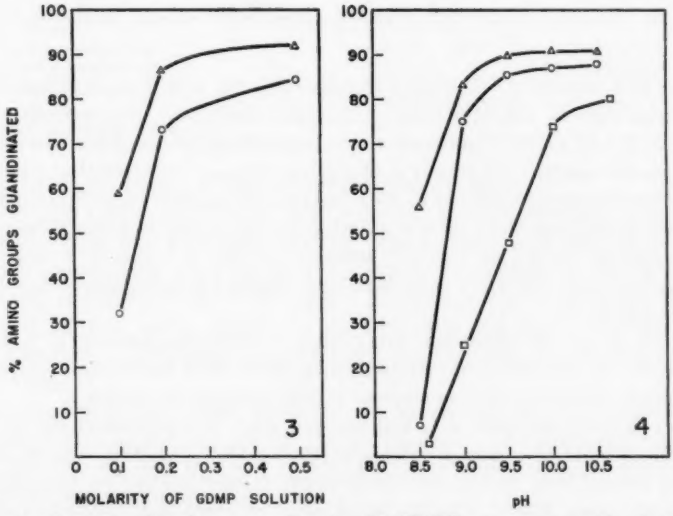

FIG. 3. Reaction of bovine serum albumin with GDMP. ○——○ reaction for 3 days; △——△ reaction for 7 days.

FIG. 4. Reaction of bovine serum albumin with GDMP. ○——○ reaction for 3 days; △——△ reaction for 7 days; □——□ results of Hughes *et al.* (3).

The influence of pH on the reaction with 0.5 *M* GDMP was studied at 0°. The results are included in Fig. 4 with results of Hughes *et al.* (3) and show that the lowest pH for maximum reaction was pH 9.5.

A study of the effect of reaction at room temperature showed that after 24 hours, 90% guanidination was reached (Fig. 5).

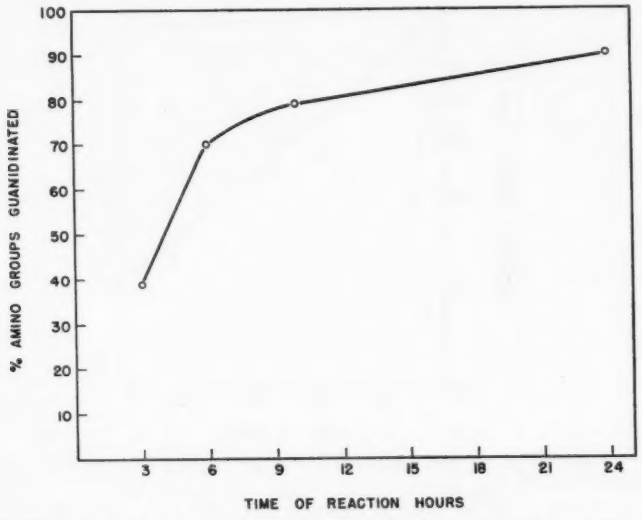

FIG. 5. Reaction of bovine serum albumin with GDMP at room temperature.

Reaction of 1-Guanyl-3,5-Dimethyl Pyrazole Nitrate with β-Lactoglobulin

The reaction of GDMP with β-lactoglobulin (LG) was complicated by gelling in 0.5 *M* GDMP solution under conditions where BSA was found to react; similar results were found in O-methyl isourea. As O-methyl isourea and GDMP are chemically related to urea and guanidine, they may cause denaturation of some proteins. Gelling was avoided in 3% protein solution if the reaction was performed in 0.2 *M* GDMP solution at pH 9.5 and 0° C for 7 days. Guanidinated LG (GuLG) thus prepared showed polydispersity upon ultracentrifugation.

Huggins *et al.* (9) observed the gelling of various proteins in 8 *M* urea, attributed it to formation of intermolecular disulphide bonds, and prevented it by sulphydryl group reagents such as *p*-chloromercuribenzoate.

To prevent aggregation, LG was reacted with N-ethylmaleimide (NEMI), iodoacetic acid (IAC), and *p*-chloromercuribenzoate (PCMB) for 30, 30, and 24 hours respectively under conditions where complete reaction of sulphydryls would take place (10). The material after dialysis and lyophilization was reacted in 0.2 *M* solution at pH 9.5 and 0° C for 7 days. GuLG prepared after treatment with PCMB showed an asymmetric peak upon ultracentrifugation, while treatment with IAC and NEMI prevented aggregation.

Reaction with Fluorodinitrobenzene

Solutions of 0.5 μmole of BSA, GuBSA, LG, and GuLG were reacted with fluorodinitrobenzene (FDNB) in 66% ethanol at room temperature as described by Fraenkel-Conrat *et al.* (11). The dinitrophenyl (DNP) derivative was hydrolyzed for 16 hours with 6 *N* hydrochloric acid and examined quantitatively for N-terminal amino acids and ε-DNP-lysine by chromatography on Whatman No. 4 paper with the ethyl benzene system (12) as developer. Optical densities of the eluted spots were measured at 360 mμ and converted to the absolute value of amino acid by means of a conversion factor. This conversion factor was obtained by subjecting the appropriate amino acid (N-terminal in the protein) to the same reaction, extraction, and chromatography as that used for the protein. The results were corrected for loss during hydrolysis (13, 14). In agreement with Porter (15) it was found that the ε-amino groups of lysine in LG were not completely reactive with FDNB except after denaturation. Therefore LG and GuLG were denatured with 66% ethanol for 24 hours before treatment with FDNB. Results are given in Table I.

TABLE I

Amino groups, ε-DNP-lysine, and N-terminal amino acid in native and guanidinated proteins

Protein	% free amino groups by ninhydrin	ε-DNP-lysine	N-terminal residue
		moles/mole protein	
Bovine serum albumin (M.wt. 65,000)	100	56.5 (55–57)*	Aspartic 0.96†
Guanidinated albumin	10	5.2	Aspartic 0.37†
Native β-lactoglobulin (M. wt. 37,300)	100	20–22	—
Denatured β-lactoglobulin	—	28 (29)*	Leucine 2.76‡
Guanidinated lactoglobulin (NEMI-treated)	10	0.7	Leucine 1.98‡

*Value in parenthesis is from literature (16, 17).
†Value corrected for 65% recovery (13).
‡Value corrected for 75% recovery (14).

Analysis of Basic Amino Acids of Guanidinated Proteins

GuBSA and GuLG (NEMI- or IAC-treated) were hydrolyzed by refluxing for 24 hours with 6 *N* hydrochloric acid. The hydrolyzates were analyzed for the basic amino acids on a 20×0.9 cm column of Amberlite IR-120 (18) at 50° C using as eluent citrate buffer (pH 5.28) containing no detergent BRIJ 35 (18). Fig. 6, *a*, *b*, and *c* show the pattern obtained from GuBSA, GuLG (NEMI-treated), and GuLG (IAC-treated) respectively. Results are summarized in Table II.

After guanidination of the proteins the appearance of homoarginine was accompanied by a decrease in the lysine content. The histidine content of GuLG (NEMI-treated) was not calculated due to interference by an unknown material with the histidine peak.

Physical Studies

A Spinco Model H Electrophoresis apparatus was utilized in electrophoresis experiments, carried out at about 0.4 – 0.5% protein concentration in barbital

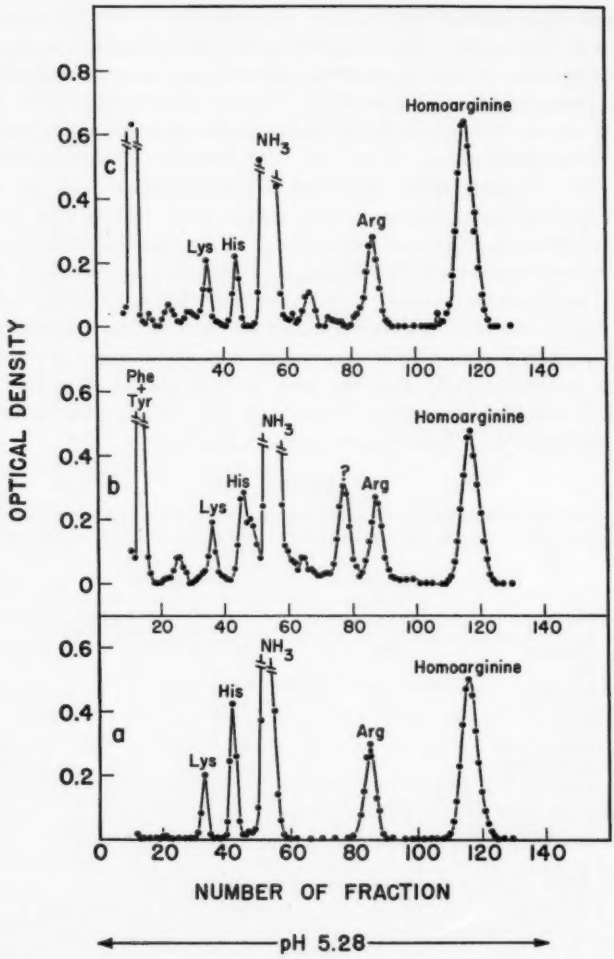

FIG. 6. The basic amino acids obtained from hydrolyzed guanidinated proteins; (*a*) GuBSA; (*b*) GuLG (NEMI-treated); (*c*) GuLG (IAC-treated).

TABLE II

Basic amino acids in native and guanidinated proteins

Amino acid	BSA	GuBSA	LG	GuLG (NEMI)	GuLG (IAC)
	moles per mole protein				
Lysine	55–57	6	28.8	1.54	1.52
Histidine	17	16.5	4.2	—	2.72
Arginine	22	22.3	6.2	6.1	6.15
Homoarginine	0	52	0	17.4	21.0

buffer pH 9 and μ 0.1. Acetate buffer pH 3.5, μ 0.1 was also used for GuBSA. Mobilities are reported for the descending boundaries.

Ultracentrifuge experiments were performed on 1% protein solutions in a Spinco Model E ultracentrifuge in phosphate buffer, pH 7.5, μ 0.1, at 59,780 r.p.m. and 20° C.

Molecular weights for GuBSA and GuLG were obtained by the Archibald procedure (19, 20) at 8225 and 12,590 r.p.m. respectively.

Results are tabulated in Table III.

TABLE III

Sedimentation coefficients, electrophoretic mobilities, and molecular weights of native and guanidinated proteins

Protein	$s_{20,w}$	Electrophoretic mobility, $cm^2/v/sec\ 10^5$		Molecular weight
BSA	4.18 S	−6.6	4.4*	
GuBSA	4.15 S	−6.6	4.7*	87,400
LG	2.41 S	−5.4		
GuLG (IAC)	1.78 S	−7.0		33,800
GuLG (NEMI)	1.79 S	—		

*Values using acetate buffer pH 3.5 μ = 0.1.

Discussion

The role of amino groups in biologically active proteins has been extensively examined by chemical modification. It is essential that no molecular configurational changes should take place as a result of chemical modification. Reagents such as succinic anhydride (1) and acetic anhydride (2) produce non-specific inactivation of antibodies because their effect on the net charge of the protein molecule caused swelling or unfolding of the protein molecule. Guanidination using O-methyl isourea avoided this change of charge near neutral pH but required an unduly high pH for the optimum reaction (3).

GDMP offers several advantages over O-methyl isourea for the guanidination of proteins. The reagent is more stable than O-methyl isourea; while 41% destruction of the latter took place after 24 hours at room temperature (21) GDMP showed insignificant loss at similar time. The slow destruction of GDMP followed a first-order reaction mechanism and was temperature dependent (Fig. 2). Guanidination of BSA was obtained by reaction for 7 days at 0° C in 0.5 *M* GDMP solution and pH 9.5, which is less alkaline than that required for reaction with O-methyl isourea (3).

In GuBSA thus prepared 5 and 6 lysine groups were unreacted as found by FDNB and by column chromatography respectively, while 52 groups of homoarginine had appeared. The sum of lysine and homoarginine was 57–58 groups in GuBSA compared to 55–57 groups (16, 17) of lysine present in BSA (molecular weight 65,000). Some lysine was probably unreacted due to steric hindrance. Hughes *et al.* (3) found that exhaustive treatment with isourea led to the reaction of 54–57 out of 64–68 amino groups determined by Van Slyke amino nitrogen analysis. Histidine and arginine were recovered quantitatively in GuBSA.

Guanidination of BSA using GDMP caused no significant configurational changes, since the sedimentation coefficients and the electrophoretic mobilities of the native and modified protein were similar.

The molecular weight, determined by Archibald's technique, was 87,400, which was higher than the usual value. This discrepancy was probably caused by the fast-moving component present in native albumin.

The results with LG were different from those with BSA and indicate configurational changes in GuLG; however, variability in the behavior of proteins towards O-methyl isourea have been reported. Guanidinated BSA (3) and ribonuclease (19) had similar sedimentation coefficients to those of the native proteins, but modified chymotrypsinogen (22), mercuripapain (23), and insulin (24) showed different sedimentation coefficients. The electrophoretic mobilities of native and modified BSA (3) and mercuripapain (23) were similar, but guanidinated chymotrypsinogen (22) exhibited a more negative mobility than the native protein.

The presence of sulphydryl groups in LG is believed to cause aggregation and gelling during the reaction using GDMP. No such difficulty was reported with O-methyl isourea when reacted with chymotrypsinogen, ribonuclease, and insulin, probably because these proteins contain no sulphydryl groups. Blocking the sulphydryl groups in LG with NEMI and IAC prevented aggregation, but PCMB was ineffective, perhaps because of the dissociation of the mercaptide bond in presence of GDMP. GuLG prepared by reaction with O-methyl isourea after PCMB treatment had a symmetrical peak on ultracentrifugation but showed aggregation as indicated by $s_{20,w}$ of 5.6 S compared to $s_{20,w}$ 2.41 S for native LG.

FDNB reaction and column chromatographic analysis of amino acids indicated 0.7 and 1.5 mole of lysine/mole protein respectively in GuLG prepared by reaction with GDMP (after NEMI and IAC treatment). The value obtained by column chromatography may be much higher than the true value since small interfering peaks (Fig. 6, *b* and *c*) appeared before and after the lysine peak. The hydrolyzate of GuLG contained several unidentified ninhydrin-positive components which may have resulted from partial destruction of homoarginine during hydrolysis and may account for the low recovery of homoarginine. Low recovery of histidine from IAC-treated GuLG was also found. Traces of IAC were found to cause destruction of some amino acids in acid hydrolysis of protein (25).

GDMP reacted with about 30% of the N-terminal leucine residues of LG and 60% of the N-terminal aspartic acid residues of serum albumin. Variability in the reactivity of N-terminal amino acids of protein towards O-methyl isourea has also been reported (22, 23, 24).

The lower sedimentation coefficient (Table III) of GuLG suggests some structural changes. The greater negative electrophoretic mobility of GuLG is analogous to the results obtained with guanidinated chymotrypsinogen (22), which was attributed to the greater binding of anions by the guanidino groups. This is supported by the fact that acetyl LG (1) had identical electrophoretic mobility as GuLG. Whereas in the former the ϵ-amino groups of lysine were

replaced by non-ionizable acetyl groups, in the latter, positively charged guanidino groups substituted the amino groups. The increased negative mobility of GuLG would be due to neutralization of the charged guanidino groups due to binding of anions. The low sedimentation coefficient of GuLG may be a result of unfolding or swelling accompanying the high net negative charge of the modified protein.

The differences in the results of the guanidination of BSA and LG emphasize the variable behavior of proteins towards a given reagent because of unique structural features. However, GDMP would be the reagent of choice for guanidination as it permits the reaction to occur under more gentle conditions than does the O-methyl isourea formerly employed.

Acknowledgments

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SOME CONSTITUENTS OF THE URINE OF NORMAL AND SCHIZOPHRENIC INDIVIDUALS

PART II[1]

R. M. ACHESON AND D. P. DEARNALEY

Abstract

A comparison has been made of the compounds detectable on two-dimensional paper chromatograms by their ultraviolet absorption or fluorescence, or by development with diazotized sulphanilic acid (DSA), in extracts of the urines of normal and schizophrenic individuals. No significant difference could be detected. Of the six DSA coupling compounds reported in Part I (1) to have a possible connection with schizophrenia, only four were detected in this survey and their distribution in the urine samples does not suggest that any have a connection with the disease. Most of the compounds reported in Part I were found again in this survey along with 70 DSA or Altman-positive new materials of which many were present in only a few samples. A small number of additional compounds were detected in the urine of schizophrenics on Chlorpromazine or Reserpine therapy. No significant difference between the human pseudocholine esterase inhibitory properties of the extracts from normal and schizophrenic urines was observed.

Introduction

A previous two-dimensional chromatographic survey (1) has been made of the compounds which give colors with diazotized sulphanilic acid (DSA) in certain extracts of urine pools obtained from normal and schizophrenic individuals living on a Canadian diet. Over 80 compounds of this type were noted, 32 were tentatively identified, and of the remainder it was concluded that only six could possibly have any connection with schizophrenia. Three of these compounds (Nos. 64, 133, and 138) occurred only in the "schizophrenic" urine pools and could therefore have had a direct relation to the disease. The other three (Nos. 59, 134, and 136) were present in the "normal" pools only, so their absence in the schizophrenic pools might have significance. The primary object of the investigation described here was to establish or discount possible connections between these six compounds and schizophrenia. Secondary aims were to look for differences between the compounds present in "normal" and "schizophrenic" urine extracts which could be detected on paper chromatograms by examination under ultraviolet light, to ascertain whether schizophrenics on Chlorpromazine or Reserpine therapy excreted additional compounds detectable by the technique employed, and see whether any difference existed between the pseudocholine esterase inhibition of the various urine extracts.

Methods

Urine Samples

Overnight urine samples, obtained from males only, were acidified to pH <3 on collection and used immediately. Samples were obtained from four patients on admission to the Warneford Hospital, Oxford. At the time, these patients were receiving no medication and were very clearly diagnosed as schizophrenics.

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Contribution from the Department of Biochemistry, Oxford University, England.

The pooled urine samples were obtained from the staff of this laboratory and from schizophrenics at St. John's Hospital, Stone. The "schizophrenic" pools were respectively obtained from 5 patients each receiving ca. 7 mg of Reserpine daily, and from 12 patients each receiving ca. 300 mg of Chlorpromazine daily. Each "normal" pool had seven contributors.

Preparation of the Urine Extracts

Each urine sample was divided into two portions. One portion (1 l.) was mechanically shaken (24 hours) with charcoal (50 g, of Baker and Adamson's, code 1551, lot M. 146), the charcoal was then collected, and thoroughly washed three times by mechanically stirring (30 minutes) with distilled water (500 ml) and filtering. The charcoal was then stirred with aqueous phenol (minimum 4 hours, 500 ml of 7%) three times at room temperature, followed by a final extraction at 50° C with aqueous phenol (4 hours, 150 ml of 25%). The combined extracts were evaporated at 35° C *in vacuo* with the addition of distilled water, until the distillate gave no color with ferric chloride, and the residue was concentrated to 41.5 ml per liter of original urine. The residual liquid was now extracted at 0 – 5° C (3) with equal volumes of ethyl acetate until the extracts were colorless. The remaining aqueous layer, which was then concentrated to 15 ml, and the ethyl acetate solution, which was similarly concentrated, were then chromatographed. At this stage it was observed that pathological samples formed emulsions much more readily than normal samples. The significance of this is not established, and it may even be due to traces of detergents left in the hospitals' urine collection vessels.

The second portion (1 l.) of each extract was treated similarly except that charcoal (50 g of B.D.H. "activated") deactivated with 6% of its weight of stearic acid (4) was used as the adsorbent and it was eluted at room temperature about three times with a mixture of aqueous phenol (250 ml, of 7%) and glacial acetic acid (50 ml), and then with 250-ml portions of a mixture of phenol (500 g), glacial acetic acid (400 ml), and water (1.5 l.) until the extracts were colorless.

Towards the end of the work the various phenolic extracts obtained from one charcoal (B.D.H. deactivated with stearic acid) were not combined but were separately examined to see if any selective elution took place. None in fact appeared to take place but it was found that only one extraction with the more concentrated phenol was necessary. Although subsequent elutions did remove a little color from the charcoal they contained almost no chromatographically detectable material.

Chromatographic Procedure

A known volume of each concentrated urine extract (approximately 0.5 ml) was left overnight in a weighed tube in a vacuum desiccator (sulphuric acid) and a known weight of the semisolid residue applied to a Whatman No. 1 chromatography paper (22.5 in. × 18.5 in.). It was then allowed to dry in air at room temperature until the residue was no longer glossy, otherwise satisfactory chromatograms were not obtained. The amount of material applied was equivalent to 10 – 20 ml of the original urine in the case of the ethyl

acetate extracts, and to 3 – 5 ml in the case of the aqueous extracts, respectively. The chromatograms were run in descending isopropanol–ammonia–water (8:1:1; 22 hours) and fresh butanol – acetic acid – water (4:1:1; 16 hours) as previously described (1) but at 12 – 14° C.

As soon as they were dry, the chromatograms were examined under a Hanovia lamp, emitting 90% of its ultraviolet radiation at 2537 Å, and the fluorescing and absorbing areas marked. They were then sprayed on the other side with diazotized sulphanilic acid (DSA) prepared at 0° C by mixing one volume of a solution of sulphanilic acid (0.9 g) in concentrated hydrochloric acid (9 ml) and diluted to 1 l. with one volume of aqueous sodium nitrite (0.05%), and 15 minutes later, three volumes of aqueous potassium carbonate (14.0%). This reagent gave a much lower background color than that used earlier and facilitated the detection of minor constituents; the other spray reagents used were as described in Part I (1).

All four extracts from each urine sample were initially chromatographed in duplicate and a careful comparison made of the compounds extracted by both types of charcoal. The charcoals did not always extract the same compounds although their over-all behavior was very similar. The most striking differences were the complete failures of the Baker and Adamson charcoal to extract compound AR, and B.D.H. deactivated charcoal to extract compounds 135 and 172 respectively. Neither charcoal was universally preferable to the other.

Results and Discussion

Figures 1 and 2 show the relative positions of the compounds located by the DSA and Altman reagents on the chromatograms of the ethyl acetate and aqueous urine extracts. Figures 3 and 4 give the corresponding data for the compounds discernible under ultraviolet illumination. Tables I – IV give tentative identification or characterization data for each compound and its incidence in the extracts. No spot is listed which did not appear on duplicate chromatograms of the same extract. If there was any doubt, further chromatograms were run and 17 spots had to be eliminated during the whole survey through failure to replicate.

The spots found here and previously recorded in Part I have been given the same numbers, which are based on those of Armstrong *et al.* (3). Where a compound, visible under ultraviolet illumination, clearly corresponded with a spot developed by DSA or the Altman reagent they were given the same number, but in the case of many unidentified compounds, particularly in the more heavily loaded parts of the chromatograms, it was impossible to be sure and then no correspondence was assumed.

Tentative identifications are only given where the R_f values and color reactions agree with those recorded earlier for known urine constituents. The general layout of the spots shown on Figs. 1 and 2 reproduces that of Acheson *et al.* (1) quite well apart from the additions and a slight over-all change in pattern. This change reaches a maximum in the region of spot No. 4 (4-hydroxyhippuric acid), the identity of which was checked by the increase in

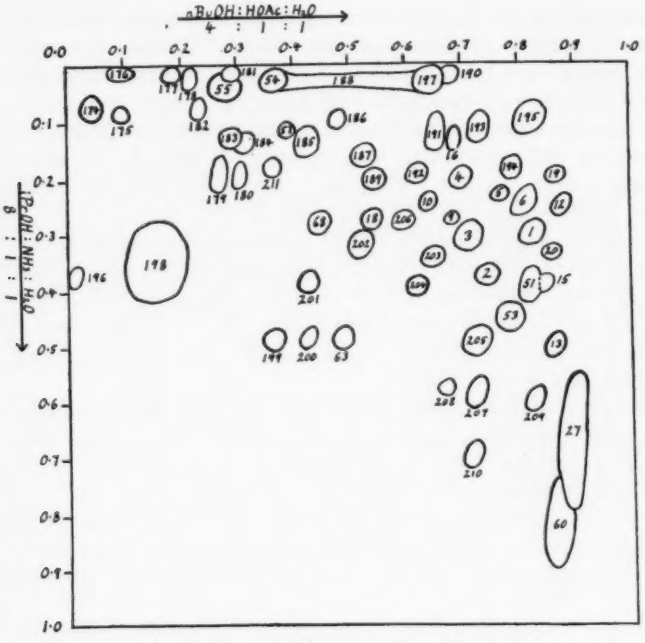

FIG. 1. Diagram of the D.S.A. and Altman spots on the ethyl acetate phase chromatograms.

intensity observed on adding authentic material, and may be due to the temperature of the chromatography room being 10° C lower than that in Vancouver.

Fifty-two of the 122 compounds detected by the use of DSA or Altman's reagent were among the 84 reported in Part I. Apart, therefore, from the compounds visible on the chromatograms only under ultraviolet illumination 70 new materials were detected.

Most of the 70 DSA and Altman positive compounds which were detected in addition to those reported in Part I were found in a minority of the urine samples, as were the 32 compounds reported in Part I but not found again here. These noteworthy differences in the minor urine constituents of Canadians and Englishmen cannot reasonably be ascribed to differences in metabolism and must therefore be due to differences in diet.

Five compounds (138, 167, CE, NW, and PE) and four compounds (158, BV, NT, and PC) only appeared in the urines of patients receiving respectively Reserpine or Chlorpromazine and are probably the drugs or their metabolites.

Compound 138 was, however, also found in all the schizophrenic urine pools, but not in the normal pools, described in Part I. It would be dangerous to conclude that the compound was therefore unconnected with the reserpine therapy for while this may be the case, as the patients providing the urine had received no tranquilizers for some weeks, some drugs including Reserpine are

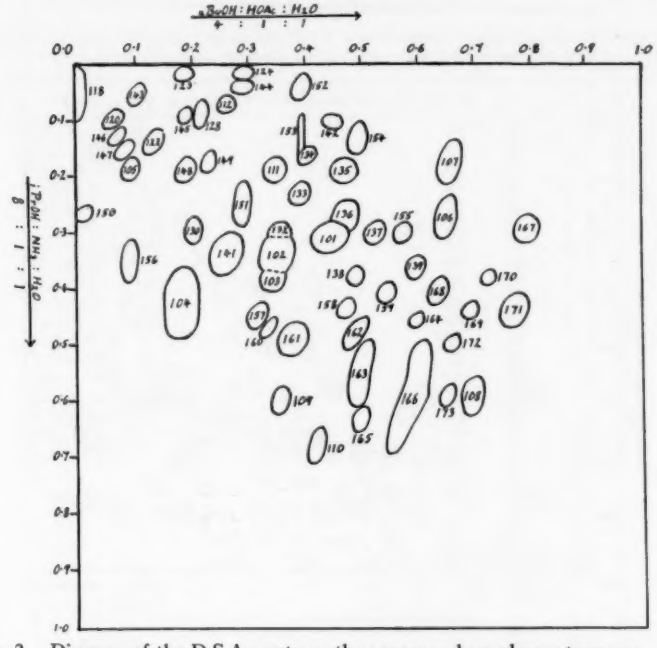

FIG. 2. Diagram of the D.S.A. spots on the aqueous phase chromatograms.

very slowly excreted. An example is Atebrin, which can be detected in human urine 69 days after the ingestion of 0.3 g (5).

Of the six compounds that were detected in Part I and which could have had a possible connection with schizophrenia, only four (Nos. 133, 134, 136, and 138) were observed in this survey and they appeared to follow a random distribution amongst the urines. It can therefore be concluded with certainty that no qualitative connection between schizophrenia and the DSA positive compounds in urine extracts can be detected by the method employed. The possibility that both qualitative and quantitative differences occur between the DSA coupling compounds in normal and schizophrenic urines has been fully discussed (6, 7). In view of the large number of DSA coupling and other compounds present in urine which are due primarily to diet and which may well vary greatly in amount it appears that observed quantitative differences between most constituents of normal and schizophrenic urines will only have significance if the diet is rigorously controlled. This could not be done in the present survey, but no clear quantitative difference was noticed in the common materials occurring in the normal and schizophrenic urine extracts.

Since the submission of this paper, a further communication (9) from Vancouver has appeared. This describes elaborations of the technique employed in Part I and the detection of further compounds, a number of which are doubtless identical with those reported here. It is to be particularly

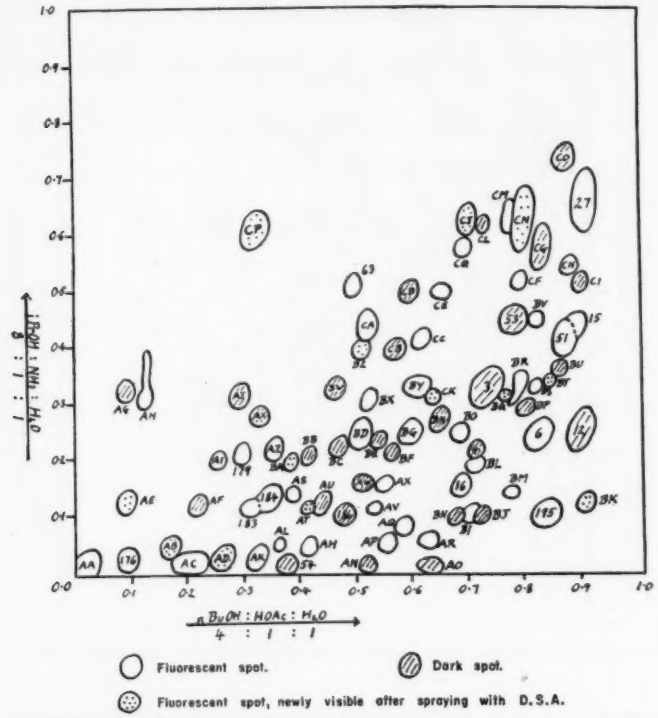

FIG. 3. Diagram of the spots visible under the ultraviolet lamp on the reverse side of the ethyl acetate phase chromatograms.

TABLE I

Characterizations, tentative identifications, and incidence of the spots developed with the D.S.A. and Altman reagents on the ethyl acetate phase chromatograms

Spot No.	Compound and (or) characterization	Amm	Ac	2N	4P	1R	1C
1	4-Hydroxyphenylacetic acid; purple	31	83	2	4	1	1
2	3-Hydroxyphenylhydracrylic acid; orange	37	75	2	4	1	1
3	3-Hydroxyhippuric acid; orange, *lt. orange*	31	72	2	4	1	1
4	4-Hydroxyhippuric acid; white, *yellow*	21	70	2	4	1	1
5	5-Hydroxyindolylacetic acid; red	23	77	0	0	1	1
6	2-Hydroxyhippuric acid; yellow, *yellow*	25	82	2	4	1	1
9	4-Hydroxymandelic acid; orange	28	69	2	4	1	1
10	4-Hydroxy-3-methoxymandelic acid; red	25	63	2	4	1	1
12	4-Hydroxybenzoic acid; yellow	25	88	2	4	1	1
13	2-Hydroxyphenylacetic acid; yellow	51	86	2	4	1	1
15	3-Hydroxybenzoic acid; yellow	38	85	2	4	1	1
16 or (17)	Feruoyl glycine; brown, *yellow* (vanilloyl glycine)	14	69	2	4	1	1
18	Orange-red	27	54	1	1	0	0
19	Vanillic acid; orange	20	86	2	4	1	1
20	Dihydroferulic acid; red	34	86	1	4	0	1
27	Salicylic acid; yellow	67	90	2	4	1	1
51	Anthranilic acid; yellow	40	83	2	4	1	1
53	Hippuric acid; white, *orange*	44	78	2	4	1	1
54	White, *purple*	02	37	2	2	1	1

TABLE I *(Concluded)*

Characterizations, tentative identifications, and incidence of the spots developed with the D.S.A. and Altman reagents on the ethyl acetate phase chromatograms

Spot No.	Compound and (or) characterization	Amm	Ac	2N	4P	1R	1C
55	Yellow, *purple*	03	29	2	2	1	0
57	Red	12	40	0	2	0	0
60	Brown	82	87	1	4	1	1
63	Yellow	49	49	1	3	0	0
68	Yellow	29	46	2	4	1	1
174	Red	06	05	0	1	0	1
175	Yellow; *grey*	08	10	2	0	0	1
176	Yellow	02	10	1	0	0	0
177	Red	03	19	0	1	0	0
178	Yellow	03	22	2	1	1	1
179	Yellow	20	28	2	1	1	1
180	Yellow	20	31	2	3	1	1
181	Yellow	01	30	1	0	0	1
182	*Red*	08	24	0	0	1	0
183	White, *yellow*	23	30	1	2	1	1
184	White, *orange*	24	33	2	3	1	1
185	Orange	24	43	1	3	1	1
186	Red-yellow	10	49	0	3	1	1
187	Yellow	26	53	2	1	0	1
188	*Pale pink*	02	37–65	2	2	1	1
189	Yellow	21	55	0	3	0	1
190	Yellow	03	67	2	2	1	1
191	Yellow	13	66	1	3	0	1
192	Yellow	19	63	2	3	1	1
193	Yellow	12	73	0	0	1	0
194	Orange-yellow	19	79	1	3	1	1
195	*Red*	10	83	2	4	1	1
196	Yellow	38	02	0	1	1	0
197	*Purple*	03	65	2	4	0	1
198	Yellow	36	16	0	1	0	0
199	Yellow	50	36	1	1	1	1
200	White	49	42	0	1	0	0
201	Yellow	39	43	1	0	0	0
202	Yellow	33	53	2	4	1	1
203	Yellow	34	66	1	1	0	1
204	Yellow	39	62	2	2	1	1
205	Yellow	49	73	2	2	1	1
206	Yellow	28	60	1	2	1	1
207	Yellow	58	73	1	2	1	1
208	Red	57	68	0	1	1	1
209	Blue	60	83	2	4	1	1
210	Brown	71	72	0	1	0	0
211	White	29	37	0	1	0	0

KEY TO TABLES I, II, III, AND IV

2N = Number of times substance occurred in two normal pooled samples.
4P = Number of times substance occurred in four individual pathological samples.
1R = Number of times substance occurred in one pooled sample from five schizophrenics under Reserpine.
1C = Number of times substance occurred in one pooled sample from 12 schizophrenics under Chlorpromazine.
Amm = R_f in isopropanol/ammonia/water: 8/1/1.
Ac = R_f in *n*-butanol/acetic acid/water: 4/1/1.
• Signifies fluorescent spot newly visible after spraying with D.S.A.

In Tables I and II the colors developed with the D.S.A. and Altman reagents are given, the latter being set in italics.

noted that differences between pools of normal and schizophrenic urine were not stressed.

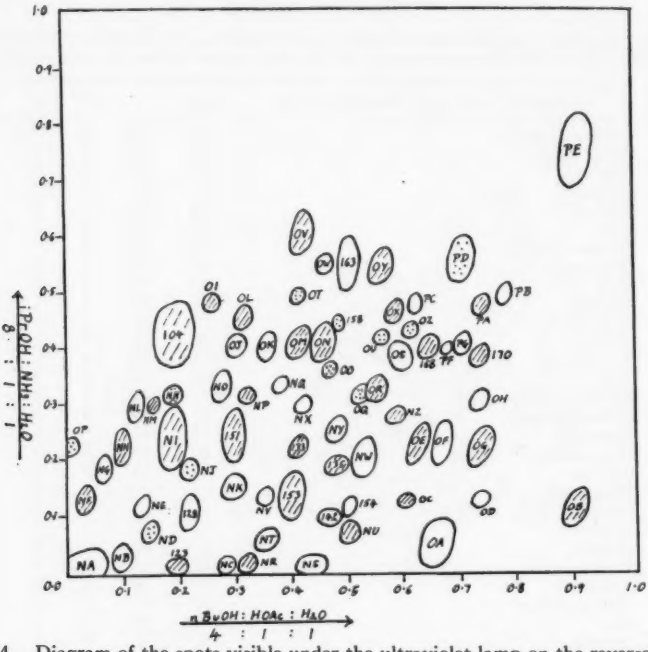

FIG. 4. Diagram of the spots visible under the ultraviolet lamp on the reverse side of the aqueous phase chromatograms.

TABLE II

Characterizations, tentative identifications, and incidence of the spots developed with the D.S.A. reagent on the aqueous phase chromatograms

Spot No.	Compound and (or)characterization	Amm	Ac	2N	4P	1R	1C
101	Tryptophan; yellow	31	45	2	4	1	1
102	Red-brown	33	35	2	4	1	1
103	Red-brown	38	34	2	4	1	1
104	Yellow or white	41	19	2	4	1	1
105	Histidine; red	19	10	2	4	1	1
106	Yellow	27	65	2	4	1	1
107	Yellow	18	66	2	2	1	1
108	Yellow	58	70	2	0	0	1
109	Yellow	60	36	1	2	0	1
110	Yellow	67	43	1	3	0	1
111	Yellow	19	35	2	2	0	0
112	Yellow	06	26	1	0	0	0
118	Red-brown	05	00	2	4	1	1
120	Yellow	10	06	2	4	0	1
122	Yellow	14	13	2	0	1	0
123	White	01	20	2	3	0	1
124	Orange-red	01	26	1	1	0	0
128	Red	09	22	1	4	0	1
130	Red	28	21	2	4	1	1
132	Red-brown	30	36	2	3	1	1
133	White	23	40	2	4	1	1
134	Red	17	40	2	2	0	1
135	Brown-yellow	19	47	2	4	1	1
136	Yellow	27	47	2	1	1	1
137	Red	30	53	1	4	0	1

TABLE II *(Concluded)*

Characterizations, tentative identifications, and incidence of the spots developed with the D.S.A. reagent on the aqueous phase chromatograms

Spot No.	Compound and (or) characterization	Amm	Ac	2N	4P	1R	1C
138	White	38	50	0	0	1	0
139	Brown	36	60	0	1	1	0
141	Red	35	27	2	4	1	1
142	Yellow	01	45	1	1	0	0
143	Yellow	05	11	0	1	0	0
144	White	03	26	0	1	1	1
145	White	09	20	2	2	0	1
146	Red	13	07	1	3	0	1
147	Red	15	08	1	3	0	1
148	Brown	19	20	1	0	0	0
149	Red	18	23	1	0	0	0
150	White	27	00	1	0	0	0
151	Yellow	25	30	0	1	0	1
152	Yellow	05	40	0	1	0	0
153	White	13	40	1	1	0	0
154	Yellow	13	50	1	0	0	0
155	Yellow	30	57	2	2	1	1
156	Red	35	10	0	1	0	0
157	Yellow	44	32	0	1	0	1
158	Green	43	48	0	0	0	1
159	Yellow	41	55	1	1	0	1
160	Red	46	35	1	0	0	0
161	Red	47	37	1	1	0	0
162	Yellow	47	47	2	3	1	1
163	Yellow	55	50	2	1	1	1
164	Brown	45	60	2	0	1	0
165	Yellow	63	50	1	0	1	0
166	Brown	58	59	2	1	1	1
167	Yellow	30	80	0	0	1	0
168	Yellow	40	64	2	4	1	1
169	Yellow	43	69	1	2	0	0
170	Yellow	38	72	1	3	1	0
171	Yellow	44	77	2	3	1	1
172	Yellow	50	66	2	1	0	1
173	Purple	59	64	0	0	1	1

TABLE III

Characterization and incidence of the spots visible under the ultraviolet lamp on the ethyl acetate phase chromatograms

Spot No.	Characterization	Amm	Ac	2N	4P	1R	1C
3	Dark	33	73	2	4	1	1
4	Dark	21	71	2	4	1	1
6	Blue	25	82	2	4	1	1
12	Dark	25	89	2	4	1	1
15	Blue	45	87	2	4	1	1
16	Blue	16	68	2	2	0	0
188	Dark	11	47	2	4	1	1
27	Blue	65	09	2	4	1	1
176	Yellow	02	10	2	2	1	1
51	Blue	41	85	2	4	1	1
53	Dark	45	76	2	4	1	1
54	Dark	02	37	2	4	1	1
63	White	50	50	0	2	0	0
179	Blue	21	30	2	4	1	1
185	Dark	13	31	2	4	1	1
186	Dark	14	35	2	4	1	1
197	Dark	11	83	1	3	0	1
AA	Blue	02	02	2	1	1	1

TABLE III *(Continued)*

Characterization and incidence of the spots visible under the ultraviolet lamp on the ethyl acetate phase chromatograms

Spot No.	Characterization	Amm	Ac	2N	4P	1R	1C
AB*	Blue	05	16	0	1	0	0
AC	Blue	02	21	1	2	0	1
AD*	Yellow	03	26	1	0	0	0
AE*	Blue	13	10	1	0	1	1
AF	Dark	12	21	0	1	0	0
AG	Dark	32	10	0	1	0	0
AH	Yellow	31	12	2	3	0	1
AI	Yellow	20	26	1	0	0	0
AJ	Dark	32	29	1	0	0	0
AK	Blue	03	33	1	4	1	1
AL	Blue	05	36	0	1	0	0
AM	Blue	05	41	0	1	0	0
AN	Dark	01	52	1	2	0	1
AO	Dark	01	63	1	4	1	0
AP	Yellow	05	55	0	1	0	0
AQ	Blue	08	58	0	2	0	0
AR	Blue	06	62	1	2	1	1
AS	Blue	14	39	2	0	0	0
AT*	Blue	12	41	1	3	0	1
AU	Dark	13	43	2	3	1	1
AV	White	12	53	1	0	0	0
AW	Dark	16	51	1	2	0	0
AX	Blue	16	54	0	1	0	0
AY*	Blue	28	33	0	1	0	0
AZ	Blue	24	35	1	1	1	0
BA*	Blue	20	38	0	1	0	0
BB	Dark	20	41	0	1	0	0
BC	Dark	23	46	1	3	1	0
BD	Blue	26	51	0	1	1	1
BE	Dark	25	54	1	1	1	0
BF	Dark	23	56	2	4	1	1
BG	Blue	25	59	1	2	0	0
BH	Dark	10	66	2	3	0	1
BI	Blue	10	70	0	0	0	1
BJ	Dark	11	73	0	2	0	0
BK*	Yellow	13	90	0	2	1	1
BL	Blue	20	71	1	3	1	1
BM	Blue	15	77	1	2	1	1
BN	Dark	27	64	0	2	0	1
BO	Blue	26	68	1	2	0	0
BP	Dark	29	79	0	1	0	0
BQ*	White	31	75	0	1	0	0
BR	Blue	35	77	0	1	1	1
BS	Yellow	34	81	1	0	0	0
BT*	Blue	34	83	0	2	0	0
BU	Dark	36	85	1	1	0	0
BV	Yellow	45	81	0	0	0	1
BW	Dark	33	46	1	3	0	1
BX	Blue	32	53	0	1	0	0
BY	Blue	32	61	0	1	0	1
BZ*	Yellow	40	41	1	0	0	0
CA	Blue	44	51	1	0	0	0
CB	Dark	40	56	0	2	0	0
CC	Blue	42	61	1	0	0	0
CD	Dark	50	59	2	4	1	1
CE	Blue	50	64	0	0	1	0
CF	Blue	52	78	0	1	1	0
CG	Dark	56	83	0	1	0	1
CH	Blue	53	87	1	2	0	1
CI	Dark	52	89	1	2	1	1
CJ*	Blue	62	70	0	4	0	0
CK*	White	31	63	0	2	0	0

TABLE III *(Concluded)*

Characterization and incidence of the spots visible under the ultraviolet lamp on the ethyl acetate phase chromatograms

Spot No.	Characterization	Amm	Ac	2N	4P	1R	1C
CL	Dark	62	72	1	1	1	1
CM	Blue	63	77	1	2	0	0
CN*	Blue	65	79	1	1	1	0
CO	Dark	74	86	1	2	0	0
CP*	Blue	60	32	1	3	1	0
CQ	White	58	69	0	2	0	0

TABLE IV

Characterizations and incidence of the spots visible under the ultraviolet lamp on the aqueous phase chromatograms

Spot No.	Characterizations	Amm	Ac	2N	4P	1R	1C
104	Dark	41	20	0	4	1	1
123	Dark	01	20	2	4	1	1
128	Blue	10	22	0	1	1	1
133	Dark	24	41	2	4	1	1
133*	Blue	24	41	2	2	1	1
135	Dark	19	47	2	3	0	0
142	Blue	10	46	1	2	0	0
151	Dark	25	30	1	2	0	0
153	Dark	14	40	2	1	0	1
154	Blue	13	50	1	0	0	0
158*	Blue	43	47	1	2	0	1
163	Blue	55	50	2	1	1	1
168	Dark	40	63	1	4	1	0
170	Dark	37	73	2	4	1	1
NA	Blue	02	05	2	4	1	1
NB	Blue	03	10	1	0	0	0
NC	Yellow	01	28	0	1	0	0
ND*	Blue	08	15	0	1	0	0
NE	Blue	12	14	2	4	1	1
NF	Dark	15	03	2	4	0	1
NG	Blue	17	06	1	1	0	0
NH	Dark	23	10	0	1	0	0
NI	Dark	25	19	2	4	1	1
NJ*	Blue	19	21	0	1	0	0
NK	Blue	15	30	2	4	1	1
NL	White	29	13	2	1	1	1
NM	Dark	30	16	0	1	0	0
NN*	Blue	31	19	0	1	0	0
NO	Blue	34	28	2	1	1	1
NP	Dark	32	31	0	1	0	0
NQ	Blue	33	38	0	2	0	0
NR	Dark	01	32	2	3	1	0
NS	Blue	01	43	0	0	1	0
NT	White	06	36	0	0	0	1
NU	Dark	08	49	1	0	0	0
NV	Blue	15	36	0	1	0	1
NW	Blue	20	52	0	0	1	0
NX	Blue	30	42	0	2	0	0
NY	Blue	26	48	1	1	0	1
NZ	Dark	28	58	1	3	0	0
OA	Blue	05	65	0	0	1	0
OB	Dark	12	89	0	1	0	0
OC	Dark	13	60	2	2	0	0
OD	White	13	73	1	0	0	1
OE	Dark	24	62	2	0	1	1
OF	Blue	24	66	0	0	1	1
OG	Dark	23	73	1	1	1	1
OH	Blue	31	73	0	1	0	0

TABLE IV *(Concluded)*

Characterizations and incidence of the spots visible under the ultraviolet lamp on the aqueous phase chromatograms

Spot No.	Characterizations	Amm	Ac	2N	4P	1R	1C
OI	Dark	49	26	1	3	0	1
OJ	White	40	30	2	0	0	0
OK	Blue	40	35	2	3	0	0
OL	Dark	46	31	0	2	1	0
OM	Dark	40	40	1	2	1	1
ON	Dark	40	45	2	4	0	1
OO*	Blue	35	47	0	1	1	0
OP*	Blue	22	01	0	1	1	0
OQ*	Blue	33	52	1	1	0	1
OR	Dark	33	55	2	3	0	0
OS	Blue	37	59	1	0	0	0
OT*	Blue	49	41	0	1	0	0
OU*	Blue	43	56	0	1	0	0
OV	Dark	57	41	0	2	0	1
OW	White	55	46	0	1	0	0
OX	Dark	47	58	2	4	1	1
OY	Dark	55	55	1	3	0	0
OZ	Blue	44	61	1	0	0	0
PA	Dark	47	73	1	1	0	0
PB	Blue	49	76	0	0	0	1
PC	Blue	47	62	1	0	0	0
PD*	Blue	56	70	0	0	1	0
PE	Blue	75	90	1	1	0	1
PF*	Blue	40	67	1	0	0	0
PG	White	41	70	1	0	0	1

Inhibition of Human Plasma Pseudocholine Esterase by Urine Extracts

Lysergic acid diethylamide (LSD-25) is among the most potent of a group of drugs which, it is said, is capable of inducing in normal human subjects a state akin to schizophrenia (for review see 8). It has also been shown that LSD-25 is an extremely potent inhibitor of plasma pseudocholine esterase (10) although it is not thought that this finding accounts for the in vivo effects of the drug. It seemed reasonable, however, to examine the urine extracts, prepared as described above, for the ability to inhibit the pseudocholine esterase of normal human plasma. Portions of the extracts used for chromatography were examined; pseudocholine esterase activity was measured manometrically by Ammon's method (2). All the extracts inhibited the enzyme; there was a considerable variation in the degree of inhibition shown by individual extracts and no significant difference between the extracts of the urines of normal and schizophrenic individuals was observed.

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